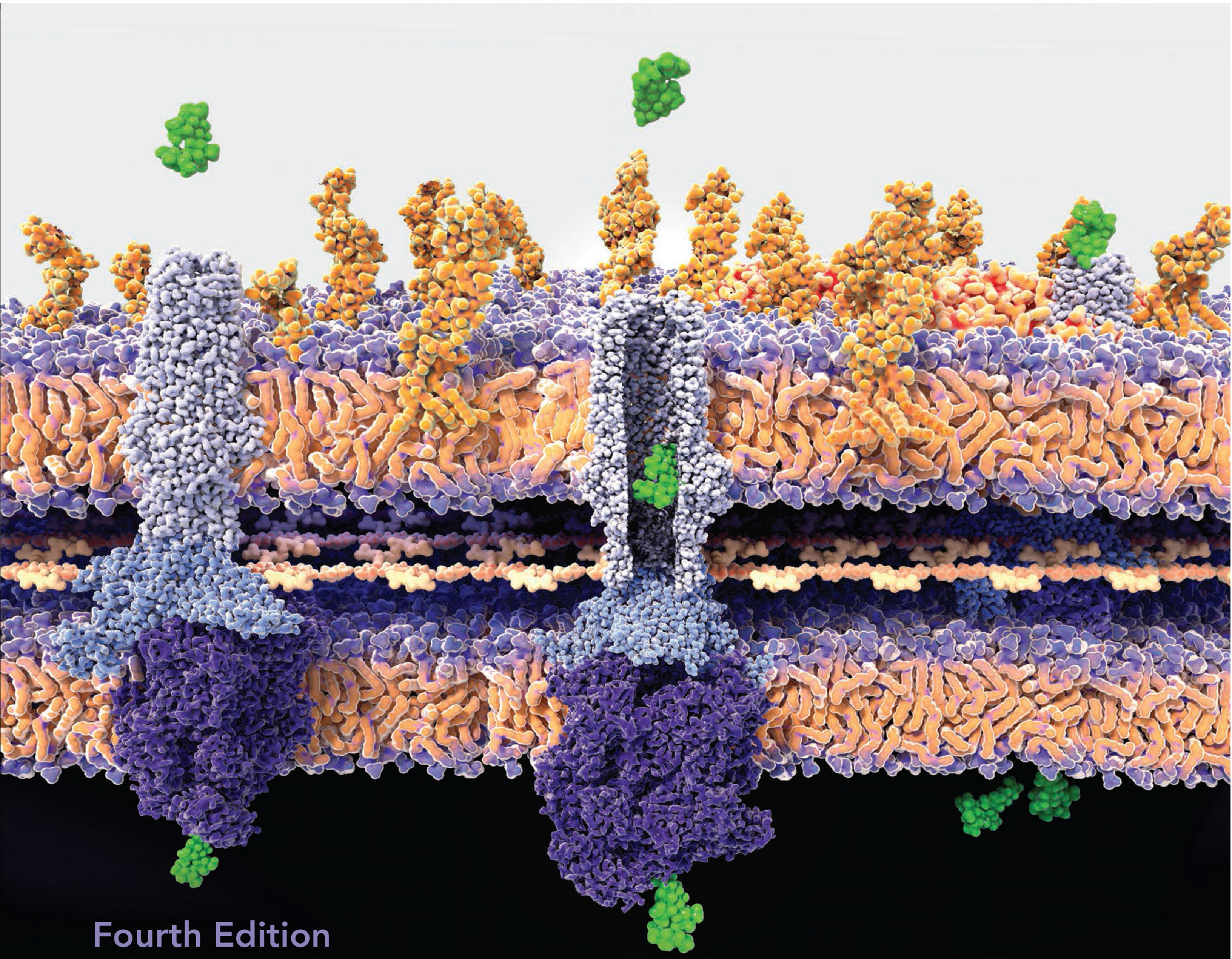

Fourth Edition

Bacterial Pathogenesis

A Molecular Approach

Brenda A. Wilson, Malcolm E. Winkler, Brian T. Ho

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A Molecular Approach

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ASM
PRESS

Washington, DC

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Library of Congress Cataloging-in-Publication Data

Names: Wilson, Brenda A., author. | Winkler, Malcolm E, author. | Ho, Brian T. (Brian Thomas), author. | Salyers, Abigail A. Bacterial pathogenesis.
Title: Bacterial pathogenesis : a molecular approach / Brenda A. Wilson, Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana-Champaign, Illinois, Malcolm E. Winkler, Department of Biology, Indiana University, Bloomington, Indiana, Brian T. Ho, Institute of Structural and Molecular Biology, University College London and Birkbeck, University of London, London, United Kingdom.
Description: Fourth edition. | Washington, DC : ASM Press, [2019] | Revised and updated edition of the work originally authored by Abigail Salyers and Dixie Whitt.
Identifiers: LCCN 2019017126 (print) | LCCN 2019018854 (ebook) | ISBN 9781555819415 (eBook) | ISBN 9781555819408 (pbk.)
Subjects: LCSH: Bacterial diseases—Pathogenesis. | Molecular microbiology.
Classification: LCC QR201.B34 (ebook) | LCC QR201 .B34 S24 2019 (print) | DDC 579.3/165—dc23
LC record available at https://lccn.loc.gov/2019017126

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Printed in Canada

10 9 8 7 6 5 4 3 2 1

doi:10.1128/9781555819415

Address editorial correspondence to
ASM Press, 1752 N St., N.W.,
Washington, DC 20036-2904, USA

Send orders to ASM Press, P.O. Box 605, Herndon, VA 20172, USA
Phone: 800-546-2416; 703-661-1593
Fax: 703-661-1501
E-mail: books@asmusa.org
Online: http://www.asmscience.org

Illustrations and illustration concepting: Patrick Lane, ScEYEnce Studios

Cover and interior design: Debra Naylor, Naylor Design, Inc.

Cover image: Antibiotic (streptomycin) molecules being expelled out of a bacterium through the TolC efflux-pump. 3d rendering. Image courtesy of Juan Gaertner/Science Photo Library, used with permission. Based on Murakami S, Nakashima R, Yamashita E, Yamaguchi A. 2002. *Nature* 419:587–593.

To Abigail A. Salyers and Dixie D. Whitt

In recognition of their intrepid vision and leadership that
forged the foundation of this fascinating exploration of
the field of bacterial pathogenesis

Herein, their legacy lives on.

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Preface

Since the third edition of *Bacterial Pathogenesis: A Molecular Approach* was written, the field of bacterial pathogenesis has vastly expanded and evolved. Some areas have been revolutionized by the manner that research is now conducted in this genome era and the wealth of information obtained from modern advanced technologies, while other areas have become obsolete. These changes have necessitated extensive updating and rewriting of many chapters of the textbook. The death of Abigail Salyers and the retirement of Dixie Whitt in 2013 necessitated the recruitment of a new author, Brian Ho, who like the other remaining authors, Brenda Wilson and Malcolm Winkler, comes with significant expertise and research experience in modern approaches to studying bacterial pathogenesis.

This textbook is intended to be a living book that evolves in response to the constantly changing research landscape of the field of bacterial pathogenesis. True to this mandate, the fourth edition maintains the overall format of the third edition, building on the core foundations and insights of the previous editions, while expanding into new concepts, key modern innovations and cutting-edge research, and new developments in the field. All-new detailed illustrations, for the first time in stunning full color, depict important concepts and mechanisms by which bacterial pathogens interact with their hosts, other microbes, and their environments, both inside and outside the body. The enormous advances that have been made in next-generation sequencing technologies and functional genomics and multi-omics approaches have necessitated a complete update for several of the chapters. Expanded topics highlight new developments in the areas of microbiomes; comparative and evolutionary genomics; the power of Tn-Seq, RNA-Seq, and gene-editing technologies; the advancing areas of autophagy, CRISPR-Cas systems, and transgenic animal models; the roles of secreted effectors and innate immunity in pathogen clearance and persistence; and the escalating challenges of antimicrobial resistance.

As with previous editions, this textbook is written for students, not for professors. Every effort has been made to maintain a student-friendly aspect to the text, which includes minimizing much of the jargon found in the field. This textbook is not intended to cover in detail every pathogen known to the medical microbiology field. Instead, there is an emphasis on presenting the material based on core underlying principles shared among pathogens and general mechanisms used by the pathogens to overcome the host defenses, and then to provide molecular approaches used by researchers to study and combat them. Each of these basic concepts is illustrated through examples using known pathogens. At the end of each chapter students can delve more deeply into the topics covered through study questions, while problem-solving questions help to hone their skills in reading, analyzing, and interpreting data, as well as designing their own experiments. A popular feature of the third edition, the research scenarios with realistic data adapted from actual laboratory studies, challenge students to interpret data and think of new experiments that could be performed to explore solutions to the problems presented. The goal of these problems is to help the students develop problem-solving skills and to gain a better understanding of the practical applications of what they are learning from the textbook. We are grateful to many of our colleagues for sharing their expertise with helpful discussions and insights that have contributed to the enhancement of the educational experience for the students through this means, including sharing a few of their favorite pathogenesis problems.

Finally, the authors would like to acknowledge how enjoyable it is to work with such a wonderful publisher as ASM Press. The authors are enormously grateful to the editorial staff at ASM Press, particularly Megan Angelini for her unparalleled competence and attention to detail during the entire writing and editing process and Christine Charlip, Director of ASM Press, for her tremendous support and for helping us keep on track. Senior Editor Greg Payne is thanked for his efforts in early development of the textbook and pushing previous editions through and for initiating this edition and bringing Christine and Megan on board. A special thanks also goes to the amazing graphics artist, Patrick Lane, who generated the wonderful diagrams and drawings for most of the figures in the textbook and who was extremely patient during the multiple rounds of edits.

In memoriam of Abigail Ann Salyers, PhD

G. William Arends Professor Emerita of Microbiology
University of Illinois at Urbana-Champaign
(December 24, 1942–November 6, 2013)

Abigail A. Salyers
1942–2013

An extraordinary, creative microbiologist and fervent advocate for public science literacy, Abigail Salyers was a powerful voice for championing the vast diversity and richness of the microbial world and expanding the realms of microbial ecology and clinical microbiology to include the varied host environments as different microbial ecosystems.

Abigail and her long-time partner in medical microbiology education, Dixie Whitt, were passionate about *Bacterial Pathogenesis: A Molecular Approach*. The importance of the groundbreaking first and second editions in coalescing the nascent field of bacterial pathogenesis was profound.

The depth of impact of having known and worked with Abigail on the third edition was enormous, and the outcome simply amazing. We are so grateful for her unwavering efforts to stretch our scientific limits, whether by tackling controversial topics, probing emerging areas, or shifting paradigms. The result is a colorful, living chronicle of this dynamic, ever-evolving field that will continue in this fourth edition and beyond.

Abigail, your “BP baby” is safe with us . . .

About the Authors

Brenda Anne Wilson, PhD, is currently a Professor of Microbiology and Associate Director of Undergraduate Education in the School of Molecular & Cellular Biology, College of Liberal Arts and Sciences, an Inaugural Professor in the Carle Illinois College of Medicine, and a Professor of Pathobiology in the College of Veterinary Medicine at the University of Illinois at Urbana-Champaign, Urbana, Illinois. She earned her BA degree in biochemistry and German from Barnard College, Columbia University, New York in 1981. She studied as a DAAD graduate fellow in biochemistry at the Ludwig-Maximilians Universität München in Munich, Germany. Dr. Wilson received her PhD degree in chemistry from the Johns Hopkins University in Baltimore, Maryland, where she received the Ernest M. Marks Achievement Award and an AAUW doctoral fellowship to study the biosynthesis of β-lactam antibiotics in the laboratory of Craig A. Townsend. She then undertook her NIH postdoctoral fellowship training with R. John Collier in the Department of Microbiology at Harvard Medical School, where she began her studies on bacterial protein toxins. Her first tenured faculty appointment was in the Department of Biochemistry at Wright State University School of Medicine in Dayton, Ohio. Dr. Wilson's current research focuses on host-microbe interactions with three main basic science and translational research thrusts. The first is understanding the structure-function, cellular activities, and molecular evolution of bacterial protein toxins and their roles in disease. The second is development of novel post-exposure antitoxin therapeutics and toxin-based therapeutic cargo-delivery platforms. The third is exploiting comparative and functional genomic technologies to explore the role and co-evolutionary host-microbe interactions of toxin-producing and extensively drug-resistant bacteria and microbiomes in health and disease.

Malcolm E. Winkler, PhD, is currently a Professor in the Microbiology Section of the Department of Biology and an Adjunct Core Professor in the Department of Molecular and Cellular Biochemistry at Indiana University in Bloomington, Indiana. He earned his BA degree in biology (biophysics) at Johns Hopkins University in Baltimore, Maryland, where he remained for his PhD degree in biology (genetics), studying the control of histidine (*his*) operon metabolic regulation by stringent response in *Salmonella enterica* serovar Typhimurium in the laboratory of Professor Philip E. Hartman. He was a Helen Hay Whitney Postdoctoral Fellow in the laboratory of Professor Charles Yanofsky at Stanford University in Stanford, California, USA, where he reported the pausing mechanism in attenuation regulation of the tryptophan (*trp*) operon and used oligonucleotides to test the *trp* attenuation model. Thereafter, Dr. Winkler was a tenured faculty member at Northwestern University Medical School in Chicago, Illinois, and the University of Texas Houston Medical School in Houston, Texas, determining the pathway of vitamin B_6 biosynthesis in *Escherichia coli* K-12 and studying the functions of tRNA modification enzymes encoded by complex operons in *E. coli*. He also worked on the physiological roles of the Hfq RNA chaperone and the post-transcriptional mechanisms that regulate MutHLS methyl-directed mismatch repair proteins in *E. coli*. Dr. Winkler next worked as a Research Advisor (Director) in antibiotic drug discovery at Eli Lilly and Company in Indianapolis, Indiana, where he changed his research program to understanding how the physiology, genetics, and cell biology of the Gram-positive human respiratory pathogen, *Streptococcus pneumoniae*, contribute to its pathogenesis and possibly reveal vulnerabilities that can be exploited as targets for new antibiotics and vaccines. Dr. Winkler's laboratory is currently determining the composition, coordination, and chronology of the multiple-protein molecular machines that synthesize the pneumococcal peptidoglycan cell wall and the homeostatic and stress pathways that regulate peptidoglycan biosynthesis. Dr. Winkler is a Fellow of the American Academy of Microbiology (AAM) and a Fellow of the American Academy for the Advancement of Science (AAAS).

Brian Thomas Ho, PhD, is a Lecturer in Bacteriology at the Institute of Structural and Molecular Biology of the University College London and Birkbeck College, University of London, United Kingdom. He began his research training as an undergraduate researcher in the laboratory of Nancy Kleckner at Harvard University, where he studied changes in chromosome dynamics throughout the bacterial cell cycle. He then went on to earn his PhD degree in the laboratory of John J. Mekalanos at Harvard Medical School, where he studied various aspects of the structure and function of the type 6 secretion system (T6SS) and its effectors in *Vibrio cholerae* and *Pseudomonas aeruginosa*. Continuing as a postdoc, his research turned toward studying the T6SS and other contact-dependent secretion systems, such as DNA conjugation, in the context of *in vivo* microbial communities. His current research focuses on understanding how underlying bacterial cell-cell interactions shape larger macroscopic microbial population dynamics and microbial community structure.

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IN THIS CHAPTER

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- Modeling the Host-Pathogen Interaction in Experimental Animals
- Correlation Studies

Selected Readings

Questions

CHAPTER 1

The Power of Bacteria

Never underestimate a potential adversary that has had a 3-billion-year evolutionary head start.

Why Are Bacteria So Much in the Public Health Spotlight Nowadays?

Widespread clinical use of antibiotics first began in the 1950s. The availability of these "miracle drugs," as they were called at the time, caused great excitement for both physicians and the public as a whole. They came at a time when the medical community was gaining greater control over infectious diseases than ever before. In clinics and hospitals, hygienic practices such as handwashing and disinfectant use were reducing the risk of disease transmission. In the community, improved nutrition made people better able to resist infections, while less crowded conditions and the availability of clean water helped reduce the spread of disease. Meanwhile, newly developed vaccines were protecting against some much-feared diseases. Despite all this, bacterial infections, such as pneumonia, tuberculosis, cholera, and syphilis, continued to take a heavy toll, and infectious diseases were still a leading cause of death. Antibiotics appeared to be the superweapon that would give humans the final decisive victory over bacteria.

In this early euphoria over the success of antibiotics, scientists and policy makers alike concluded that bacterial infections were no longer a threat and turned their attention to other problems, such as cancer, heart disease, and viral infections. For the next three decades, bacteria were of interest mainly as tractable model systems for studying physiology, genetics, and ecology, and as a source of tools for the new molecular biology and genetic engineering technologies that were revolutionizing all of biology. Confidence that bacterial diseases were completely under control was bolstered by a glut of new antibiotics on the market. Indeed, there was a pervasive perception among the medical community and the public as a whole that bacterial infectious diseases were no longer a problem since they could now be readily and effectively treated with antibiotics.

Unnoticed by all but a few researchers in the field and some pharmaceutical companies, the first cracks soon began to appear in the protective shield against bacterial diseases. This danger became more evident in the late 1970s. Antibiotics were no longer the highly profitable products they had once been, especially not compared to heart medications or tranquilizers, which needed to be taken daily for long periods of time. Additionally, new antibiotics were becoming harder to discover and more expensive to develop. One pharmaceutical company after another quietly cut back or dismantled its antibiotic discovery program. For a while, these cracks appeared not to matter, as there were enough new antibiotics that still worked on the bacteria that had become resistant to the old standbys like penicillin. Warnings from scientists that bacteria were becoming more resistant to antibiotics were largely ignored.

During the late 1980s, however, scientists and health officials began to notice an alarming increase in difficult-to-treat bacterial infections. By 1995, infectious diseases became one of the top five causes of death in the United States. Even with the AIDS epidemic in full swing, most infectious disease deaths were still caused by bacterial diseases, such as pneumonia and bacterial bloodstream infections (**sepsis**). Why was the incidence of bacterial pneumonia and sepsis increasing? For one, the population as a whole was aging, and older people are more susceptible to these diseases. For another, modern medicine had created a large and growing population of patients whose immune systems were temporarily disrupted due to cancer chemotherapy or immunosuppressive therapy following organ transplants, as well as due to other immune-compromising illnesses such as AIDS.

A development that caught many in the medical community by surprise was the appearance of new diseases that were dubbed **emerging infectious diseases**. In the past, scientists had assumed that any microorganism capable of causing disease would surely have done so by now, given the hundreds of thousands of years humans have occupied the planet. This view overlooked two important facts. First, bacteria can very rapidly change their genetic makeup to take advantage of new opportunities. Members of some bacterial populations are hypermutable, allowing them to try many genetic combinations to find the one most appropriate for the current environment experienced by that bacterium. Many bacteria can also acquire genes conferring new virulence traits or resistance to antibiotics from other related or even unrelated bacteria through a phenomenon known as **horizontal gene transfer (HGT)**. Second, changing human practices, such as increased global travel, widespread use of air-conditioning, and the appearance of crowded intensive care wards in big hospitals, brought susceptible people into contact with microorganisms that had not previously had the opportunity to cause human infections.

A new category of disease-causing bacteria was recognized: **opportunistic pathogens.** These bacteria normally do not cause disease in healthy people, but can infect and cause disease in individuals whose defenses are compromised in some way. In fact, many of these pathogens are found normally in or on the human body and thus had been assumed to be innocuous. Others are bacteria commonly found in soil or aquatic environments. During the early antibiotic era, these soil bacteria were thought to be beneficial to humans because scientists were finding that many of them were producers of antibiotics. However, these bacteria were suddenly being seen as the only bacterium isolated from the blood, lungs, feces, or wounds of seriously ill patients. They also tend to exhibit a troubling, not-so-friendly characteristic. Because of the antibiotics present in their natural environment, they are often intrinsically resistant to a variety of antibiotics, which made infections by these opportunistic bacteria challenging to treat.

Scientists and physicians reluctantly began to realize that a decisive human victory over bacteria had not occurred. Not only were known pathogens changing to be more resistant to antibiotics or better able to cause disease, but also new pathogens with markedly different virulence traits were emerging. The infectious disease picture was changing in a way that made it increasingly difficult to predict new patterns of bacterial disease. The serious threat of antibiotic-resistant bacteria to human health both in the United States and the world was documented in 2013 by reports from the **Centers for Disease Control and Prevention (CDC)** and the **World Health Organization (WHO)**.

Bacteria, a Formidable Ancient Life Form

The brief foregoing account of how bacterial diseases have come into prominence as a major global health problem explored the recent past. However, to understand fully why no one should have been surprised by this development and why bacteria are such formidable opponents, it is necessary to take a closer look at the long history of bacteria, during which they were constantly forced to evolve and adapt to new conditions.

Today, we realize that Earth is a microbial planet. Bacteria were probably the first form of life to appear on Earth, about 3.5 to 4 billion years ago (Figure 1-1). Bacteria and another type of prokaryote, the archaea, ruled the world unchallenged for at least a billion years before the first eukaryotes appeared. During this period, they helped create the global geochemical cycles that made Earth habitable for larger life forms. Bacteria and archaea are master recyclers. Bacteria put the first molecular oxygen in Earth's atmosphere, thereby creating the ozone layer, which protected Earth's surface from the deadly radiation that had previously bombarded it, making life on Earth's surface possible.

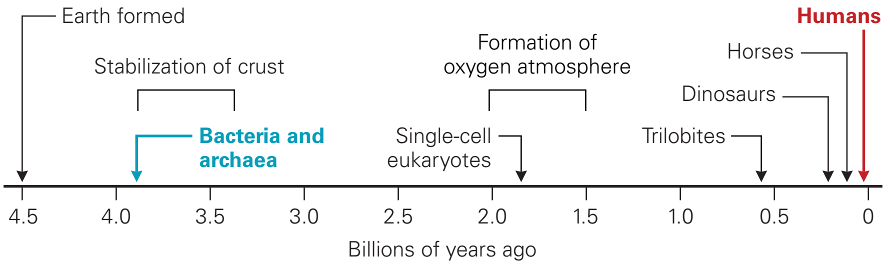

Figure 1-1. Overview of microbial evolution. Microorganisms appeared 3.5 to 4 billion years ago and changed Earth such that eukaryotes could evolve.

By adding molecular oxygen to the atmosphere, bacteria created conditions that permitted the later evolution of oxygen-utilizing creatures, including humans.

In the course of their long history, bacteria developed a variety of metabolic capabilities that allowed them to survive under an impressive variety of conditions. There are bacteria that can obtain energy by oxidizing sulfides, reducing sulfate, oxidizing ammonia, reducing nitrate, and oxidizing methane—to name only a few of the vast number of metabolic types represented in the bacterial world. Bacteria also learned how to maximize the plasticity of their genomes, constantly acquiring new DNA and mutating or rearranging existing genes, thereby creating new capabilities that enabled them to colonize the many niches on Earth. So far, no part of Earth has been found to be free of bacteria and archaea. They are in arctic ice, the deep subsurface of landmasses, the surfaces and depths of the oceans, and boiling hot springs. The genetic plasticity that enabled evolution of such metabolically diverse organisms continues to serve bacteria today as they face new challenges and opportunities.

About a billion years after bacteria first appeared on Earth, the first **eukaryotes**, the single-celled **protozoa,** emerged. Disease-causing bacteria are often capable of doing so because they have developed strategies for evading **phagocytosis** (engulfment) or for surviving inside phagocytic cells. Evolution of such strategies could have begun soon after the appearance of the first protozoa, well in advance of the appearance of animals and humans. Some of the toxic proteins that disease-causing bacteria use to kill human cells might have originally evolved to allow bacteria to evade or survive feeding by their protozoal adversaries. Today, scientists are finding that some bacterial pathogens that are harmful to humans normally live inside amoebas in nature. If this view of bacterial evolution is correct, then there are likely to be more unidentified disease-causing bacteria in nature than we currently know.

As bacteria evolved diverse metabolic specialties, acquisition of bacteria or archaea as coinhabitants (**endosymbionts)** enabled eukaryotes to expand their metabolic diversity as well. Eukaryotes acquired bacterial endosymbionts (now known as **mitochondria**) that allowed them to gain energy through respiration and to regulate cellular metabolism, thereby also enabling them to become multicellular. Cells of what would later become plants acquired photosynthetic bacteria as endosymbionts (now known as **chloroplasts**), again conferring on them the ability to harness energy from the sun through photosynthesis. Some plants went even further and also recruited into their root cells prokaryotic endosymbionts (both bacterial and archaeal), which could fix atmospheric nitrogen into nitrogenous compounds that the plant could then use as fertilizer.

As animals and eventually people emerged, bacteria were quick to take advantage of the warm, moist environment offered by their intestinal tracts. In turn, many of these bacteria helped to provide nutrients for the animal or human host by metabolizing the intestinal contents. There is no point in a human life span, except for the brief time the fetus spends in the uterus, during which the human body is not heavily colonized by large numbers of bacteria, especially on the skin, in the mouth, in the intestinal tract, and in the vaginal tract. These bacteria are highly adapted to their niches in and on the human body. However, their constant presence puts them in a position to take advantage of any breach in the defenses protecting the interior tissues and bloodstream from bacterial invasion.

Although some eukaryotic microbes such as algae have a photosynthetic lifestyle, many others, especially amoebas and other protozoa, live by feeding on bacteria and archaea. An interesting aspect of this protozoal grazing that has attracted particular attention recently is that protozoa have properties that are remarkably similar to human **phagocytic cells**, cells that form an important part of the defenses of the human body. Some of these human phagocytic cells function mainly to engulf, break down, and clear bacteria in blood and tissues. Of these, some then present components of broken-down bacteria to the cells of the immune system.

When animals and humans finally appeared on the evolutionary scene, bacteria immediately took advantage of them as rich niches in which to grow. To a bacterium accustomed to the vagaries of the external environment, where ambient temperature and availability of water and nutrients can vary widely (and unpredictably), a warm-blooded animal whose body temperature is stably maintained and whose lifestyle involves constantly collecting food and water from the environment must be as close as it gets to bacterial heaven. As such, it should not be surprising that the bodies of humans and animals carry dense bacterial loads, especially in the mouth, intestinal tract, and vaginal tract. Small wonder that the human or animal body is often referred to in the scientific literature as the **host** and that the interaction between a bacterium and its host is referred to as a **host-microbe relationship.**

Until recently, scientists studying the evolution of insects, animals, and humans almost completely ignored the selective pressure exerted by the long-term presence of the large and diverse populations of bacteria with their hosts. Now, a rapidly expanding area of research has emerged that is devoted to studying the coevolution of hosts with their resident microbial communities, also known as **microbiomes**. As will become evident in the next few chapters, the effects of microbial pressure can be seen clearly in the design of human skin, eyes, lungs, intestinal tract, vaginal tract, and in particular the immune system. Overwhelming evidence points to the vital role that microbiomes play in both promoting health and modulating disease susceptibility and severity. For the first time, we are also realizing the importance of considering the ancestry of both the host and the microbe when considering pathogenic potential (Box 1-1).

Box 1-1.

Sharing an Ancestral Relationship with Your Resident Pathogen Can Prevent Stomach Ulcers and Cancer

After more than two decades of controversy and intense experimental investigation, it is now well-established that *Helicobacter pylori*, a bacterium that colonizes the stomach of nearly half of the world's population, is the leading cause of gastric inflammation, which can lead to stomach ulcers and in a small percentage of infected individuals (<1%) stomach cancer. Extensive phylogenetic evidence suggests that *H. pylori* bacteria are about as old as modern humans and that since leaving Africa with their hosts these bacteria have diversified geographically in parallel with their human hosts. But until recently, researchers had a hard time correlating the prevalence of *H. pylori* infections with the incidence of cancer.

The first clue that other factors were at play came from comparing disease prevalence in two populations of Colombians, located about 200 kilometers from each other, with similar levels of *H. pylori* infection. In this study, the researchers compared *H. pylori* strains colonizing a coastal population of largely African ancestry (58%) having a low incidence of gastric cancer (~6 per 100,000) with those from a mountain population of mostly Amerindian descent (68%), in which gastric cancer is more common (~150 per 100,000). The results showed that Colombians of African ancestry infected with African bacterial strains had low incidence of disease, while Colombians of Amerindian ancestry infected with African strains had a high risk of cancer. Indeed, the more Amerindian ancestry an individual had and the more African-like the *H. pylori* strains harbored by that individual were, the more likely that person was to have severe gastric disease.

So, it looks like the longer you have coevolved with your resident pathogen, the less likely you are to progress to a diseased state. These findings further support a popular theory of pathogenesis that chronic pathogens that spread through vertical transmission from parent to child are predicted to become less virulent over time as a consequence of coevolution.

Sources:

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Kodaman N, Pazos A, Schneider BG, Piazuelo MB, Mera R, Sobota RS, Sicinschi LA, Shaffer CL, Romero-Gallo J, de Sablet T, Harder RH, Bravo LE, Peek RM Jr, Wilson KT, Cover TL, Williams SM, Correa P. 2014. Human and *Helicobacter pylori* coevolution shapes the risk of gastric disease. *Proc Natl Acad Sci USA* **111:**1455–1460.

Pressing Current Infectious Disease Issues

Having made this digression into ancient history, let us now return to the present and examine some bacterial infections that are at the forefront of burning public health issues. These include **emerging infectious diseases**, increasing problems with large outbreaks of **foodborne** and **waterborne infections**, **hospital-acquired (nosocomial) infections, disease transmission**, **antibiotic resistance**, **microbiota shift diseases**, **pathogen evolution,** and **bioterrorism**.

Emerging and Reemerging Infectious Diseases

The emergence of apparently new bacterial diseases and the reemergence of old diseases thought to be under control (at least in developed countries) was an unpleasant shock to the health care community. Emerging and reemerging infectious diseases illustrate an important principle. Disease patterns change, both because bacteria change by acquiring new traits through genetic mutation and horizontal gene transfer and because changing human activities can create new opportunities for bacteria to cause disease.

Not all diseases are truly emerging in the sense of being completely new to the human population. In some cases, the disease symptoms have been around for a long time as a known disease, but the bacterial cause has only recently been identified. A good example of this phenomenon is gastric ulcers, which are now known to be caused largely by the bacterium *Helicobacter pylori*. This bacterium eluded notice previously because the methods for cultivating and identifying it had not yet become common practice and because many medical researchers were convinced that no bacteria could colonize the human stomach. In this sense, many of these diseases are "emerging" in terms of public awareness but not in the minds of the scientists who study them.

Old diseases can return if the conditions change to favor their reemergence. For example, the increased use of antibiotics in hospital settings with inadequate stewardship has led to known pathogens, such as *Clostridium difficile*, *Klebsiella pneumoniae, Streptococcus pneumoniae,* and methicillin-resistant *Staphylococcus aureus* (MRSA), acquiring new antibiotic-resistance traits that now challenge measures to control their spread. Unfortunately, noncompliance with hygiene practices by health care workers promotes the spread of these bacteria among susceptible hosts.

As another example, tuberculosis made a comeback in developed countries, such as the United States and Europe during the 1990s. Its reemergence was due to several causes. One was the dismantling of the preventive infrastructure that had been developed in the 1950s to contain the spread of this insidious disease. Another was the presence of unprecedentedly large populations of highly susceptible individuals in crowded settings, such as prisons and homeless shelters. Further complicating matters were the emergence of other illnesses, such as AIDS, that suppress the immune system of afflicted individuals and the development of resistance to the traditional anti-tuberculosis drugs, which had not been updated or improved since their original introduction because no one thought tuberculosis would return.

Foodborne and Waterborne Infections

Many foodborne and waterborne infections fit into the category of emerging or reemerging infectious diseases. We treat this subject as a separate category because of its unique impact on the public perception of disease risks. Ironically, as food and water supplies have become cleaner, the public's concern about their integrity has increased rather than decreased. A review of news articles from the past few years makes this quite evident. The integrity of the food and water supplies is a nonnegotiable issue as far as the public is concerned.

From the 1960s through the 1980s, the public's main concern about the food supply was pesticide residues and other chemical adulterants that might cause cancer. This problem has been largely solved by more stringent regulations and testing, which limit the amount of pesticide residues and other harmful chemicals that might be found in food sold for human consumption. More recently, concern has arisen about another hazard that had been around all along but had not been perceived as a threat: **foodborne bacterial diseases.**

The catalyst for the abrupt swing in public concern regarding food safety was *Escherichia coli* O157:H7, a type of *E. coli* that can cause kidney failure and death, especially in children. Widespread media attention was first drawn to this problem in 1993, when an outbreak of disease was caused by undercooked, contaminated hamburger meat dispensed by an American fast-food chain. Although there had been prior outbreaks in the previous decade, many in the beef and fast-food industries and most of the general public had never heard of *E. coli* up to this point. In total, this outbreak involved 732 cases, including 4 deaths, from 73 restaurants in a number of western states before the source of the outbreak was identified and the contaminated meat was recalled. Since then, there have been many more cases of *E. coli* O157:H7 infections spread by undercooked meat, radish sprouts, lettuce, spinach, and even apple juice. The apple juice incident, during which 65 cases with 1 death were reported, nearly bankrupted the company that had produced the contaminated juice, which had not been pasteurized. The lesson that juice was not exempt from bacterial contamination was learned very quickly by the industry, and it is now rare to see unpasteurized juices in supermarkets.

Earlier in the 20th century, before the advent of centralized food processing and distribution, foodborne disease outbreaks tended to be confined to church socials, family gatherings, or business-sponsored employee picnics (Box 1-2). As the food industry became more centralized, however, a different pattern of foodborne disease emerged: the multistate (or even multicountry) outbreak of foodborne disease derived from a single source. The nature of foodborne outbreaks has changed considerably from the days of the church social outbreaks, and the effect on the public has been dramatic. In the case of the *E. coli* O157:H7 outbreak described above, a single processing plant was the source. Contaminated radish sprouts caused another outbreak of *E. coli* O157:H7 in Japanese schoolchildren. The seeds used by the Japanese sprouting companies came from a single source in the northwestern United States, where the initial contamination event probably occurred. In 2006, *E. coli* O157:H7-contaminated spinach grown in California and used by consumers in spinach salads was spread throughout the United States. In 2009, *E. coli* O157:H7 showed up yet again, this time in refrigerated cookie dough sold throughout the United States.

Similar to foodborne diseases, outbreaks of **waterborne disease** continue to occur, despite advances in water management and sanitation; however, they tend to be more localized in nature. Most of the waterborne outbreaks that have made the news lately were caused by aging pipes and water treatment plants or by mammal and bird fecal material in water reservoirs. Over half of all waterborne illnesses in the United States in recent years have been associated with contamination of drinking water with *Legionella*, a Gram-negative bacterium commonly found in soil and aquatic environments. Other microbes associated with waterborne outbreaks are *E. coli* and *Campylobacter* bacteria and the parasites *Giardia* and *Cryptosporidium*. The media picked up on this contamination problem, as was seen on a cover of *Time* magazine (August 3, 1998) showing *E. coli* cascading out of a kitchen tap. The event that stimulated this particular media response was an outbreak that occurred among 61 residents of Alpine, Wyoming (total population 470), who drank tap water on one particular weekend. Wild animals had probably contaminated the spring that was the source of the town's tap water.

An often-overlooked aspect of waterborne infectious microorganisms is that contaminated water can also produce a contaminated food product if that water is used to wash the food. In most cases, water used to wash dirt off fruits and vegetables prior to shipping is not tap water quality and is instead what is referred to as "gray water": water processed to remove the worst contamination but not microbiologically sterile. In some foodborne outbreaks, the contamination may actually have come from water used to wash the food. At first, vegetarians felt safe because foodborne diseases were so often spread by meat. But nowadays most people concerned with ensuring food safety consider any foods that are consumed raw, such as fruits and vegetables, a potentially more serious threat to public health. If meat or other food is properly cooked, the contamination problem is solved, but even careful washing is not always sufficient to render contaminated raw fruits or vegetables safe.

Box 1-2.

Bioterrorism Hits Oregon Salad Bars

In 1984, a large outbreak of salmonellosis involving at least 750 people occurred in The Dalles, the county seat of Wasco County in Oregon. At the time, a religious commune was at odds with local residents over land-use restrictions placed on the commune in an attempt by the townspeople to eliminate it. Members of the commune felt that the outcome of an upcoming election was critical to their future ability to grow. In an apparent attempt to disrupt the election, commune members planned to cause an outbreak of salmonellosis that would keep people home from the polls. The outbreak was a trial run to determine the best way to create the most havoc. At least 10 restaurants were involved, with the salad bar being the main site of intentional contamination. Contamination attempts were also made at local grocery stores, but the restaurant salad bars were the most effective source of disease. Unfortunately for commune members, their trial run was too successful and attracted the attention of the Public Health Department and the police.

Even so, it took nearly a year to trace the epidemic source and to suspect intentional contamination. Such events are fortunately quite rare, so intentional contamination was not even considered at first as a possible explanation for the outbreak. Careful questioning of the victims led investigators to deduce that salad bars had been the main source of the outbreak. Further interrogation of commune members by police and FBI agents revealed that the commune members had been the perpetrators of the outbreak. The commune had its own laboratory, where the strain of *Salmonella enterica* serovar Typhimurium was grown and prepared for inoculation of the salad bars. Commune members had apparently gotten the strain by ordering it from the American Type Culture Collection (ATCC), a widely respected repository of bacterial strains that distributes strains to scientific laboratories for a modest fee. Nearly 2 years after the outbreak, two commune members were sentenced to 1 to 2 years in prison for conspiring to tamper with consumer products. An earlier episode of product tampering involving introduction of cyanide into Tylenol capsules had been responsible for a rash of antitampering legislation. These antitampering laws were used to prosecute the commune members. As a precaution against misuse of its cultures, the ATCC has tightened restrictions on obtaining certain bacterial stocks.

Source:

Török TJ, Tauxe RV, Wise RP, Livengood JR, Sokolow R, Mauvais S, Birkness KA, Skeels MR, Horan JM, Foster LR. 1997. A large community outbreak of salmonellosis caused by intentional contamination of restaurant salad bars. *JAMA* **278:**389–395.

Modern Medicine as a Source of New Diseases

Modern medicine has made impressive breakthroughs in therapies for many human diseases. Surgeons now routinely transplant new organs into patients whose own organs are failing. Cancer chemotherapy is becoming more and more effective. This progress has come at a cost, however. Transplant patients and patients receiving cancer chemotherapy have suppressed immune systems due to the medications they are taking. This immunosuppression is temporary in the case of cancer patients and ends when the chemotherapy is finished, but transplant patients take their immunosuppressive therapy for life. Also, there are a large number of individuals suffering from infectious diseases, such as **human immunodeficiency virus (HIV)**, that are immunosuppressive. Not surprisingly, these immunocompromised patients can become infected with bacteria never before suspected to be able to cause human disease.

Other bacteria cause disease not because the patient is immunocompromised, but because a physical barrier against bacterial invasion is bypassed. For example, accidental perforation of the colon during surgery releases gut bacteria into tissue and blood. Patients with certain types of respiratory infections may have ventilator tubes inserted to keep their airways open, which can allow bacteria to bypass some of the defenses of the respiratory tract to directly enter the lungs. Likewise, catheters enable bacteria to bypass the normal cleansing flow of urine to gain access to the bladder.

Opportunistic pathogens that normally do not cause disease are able to cause disease because some defense of the body that normally keeps them at bay has been breached, giving them the opportunity to cause infection. For an interesting example of an opportunist from an unexpected source, see Box 1-3. The term "opportunist" makes such bacteria seem somehow less dangerous than "real" disease-causing bacteria. Do not be fooled by the seemingly innocuous na-

Box 1-3.

Enterprising Bacteria Always on the Alert for New Infection Opportunities

An example of how bacteria can rapidly act to take advantage of new opportunities is provided by an outbreak of pneumonia in an intensive care ward. Many of the patients were very ill and were on respirators to support breathing. The type of respirator being used required that a tube be inserted deep into the airway of the lungs, an ideal conduit to carry bacteria deep into the lung, bypassing the normal respiratory defenses. After a number of previous cases of respirator-associated lung infections, hospital personnel have learned to be very careful not to contaminate the respirator itself or to allow bacterial contaminants to enter the air being forced into the lung.

No one, however, thought about mouthwash. Since patients on respirators are often unable to attend to their own dental hygiene, hospital staff workers use mouthwash to clean and freshen the mouth every day. The cause of the lung infections was identified as *Burkholderia* (formerly *Pseudomonas*) *cepacia*. Although this bacterial species is known to cause infections in people with lung diseases, such as cystic fibrosis, it is generally considered to be a relatively innocuous soil bacterium. In fact, *B. cepacia* is used as a biocontrol agent to degrade herbicides, such as 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, a toxic defoliant also known as Agent Orange). The bacterium is ubiquitous in soil and water, but in this case apparently managed to contaminate many lots of the mouthwash, which did not contain alcohol to discourage bacterial growth. In effect, the hospital workers taking care of the patients on respirators were inoculating the patients' teeth and gums daily with a contaminated mouthwash solution, which placed the bacteria in an ideal location to gain access to the lungs.

Source:

Centers for Disease Control and Prevention (CDC). 1998. Nosocomial *Burkholderia cepacia* infection and colonization associated with intrinsically contaminated mouthwash—Arizona, 1998. *MMWR Morb Mortal Wkly Rep* **47:**926–928.

ture of the opportunists! In most developed countries, a person is far more likely to die from an opportunistic infection than from the epidemic diseases that serve as the public's mental image of infectious diseases.

Another way in which modern medicine has affected the infectious disease picture is by increasing the human life span. The increasing number of elderly people, whose immune defenses are beginning to decline and who are more likely to be receiving therapies that undermine the defenses of their bodies, provides an expanding population of individuals highly susceptible to diseases. Put these elderly people in crowded conditions, such as those experienced in nursing homes, and an even greater opportunity is created for infectious diseases to spread.

Postsurgical and Other Wound Infections

Most recent studies of wound infections have focused on the infections that can be a serious complication of surgery (**postsurgical infections).** In the preantibiotic era, infections were a major complication of surgery. Regardless of how skillful the surgeon, an infection could kill the recipient of the most successful surgery. This may have been the origin of the grim old joke that the surgery was a success, but the patient died. Antibiotics changed all this and made routine surgery possible because antibiotics eliminated any bacteria that might have managed to penetrate the barrier of the surgical scrub and other hygienic procedures.

The first shadow in this rosy picture appeared when surgeons and other health care workers began taking patient survival for granted and became more lax in their time-consuming hygienic practices. Hospitals trying to save money began cutting budgets for nurses and janitors, individuals responsible for the cleanliness characteristic of hospitals in developed countries. To make matters worse, the bacteria often causing postsurgical problems tend to be resistant to antibiotics. These postsurgical infections have consequences for both patients and hospitals. Patients risk damage to major organs or even death, while hospitals and insurance companies bear significantly higher financial costs to care for these patients.

Not long ago, the state of Pennsylvania made history by publishing postsurgical infection data from its hospitals. Until this unprecedented move to transparency, infection rates in hospitals were secrets guarded almost as fiercely as classified CIA files. The reason is easy to understand. No hospital wants potential users of its facilities, especially people getting elective surgery, to identify the hospital as a place where people go in healthy and come out sick or even dead. The Pennsylvania figures confirmed what everyone in the infectious disease community already knew: patients who contract a postsurgical infection, especially one caused by antibiotic-resistant bacteria, cost over four times more to treat than people who do not contract an infection. Unfortunately, this type of statistic has attracted a lot more attention than the suffering of the patients involved. The good news is that this increased transparency has led to improved antibiotic stewardship and hygiene practices, which in the long run will help individuals who go into hospitals.

Recently, however, attention has once again focused on another old problem: war-related infections. Accounts of the antibiotic revolution often point out how a combination of antibiotics and improved surgical interventions enable the treatment of wound infections that once killed soldiers more frequently than the trauma of the wounds themselves. World War I was the last war in which infectious diseases—not just wound infections but also diarrhea and pneumonia—were the main cause of soldiers' deaths.

An ominous development in the past decade or so has been the appearance of a soil bacterium, *Acinetobacter baumannii,* first as a wound infection problem in soldiers and now as a dangerous hospital-acquired infection. *A. baumannii* was not a stranger to microbiologists, as there had been a few outbreaks in intensive care wards, but *A. baumannii* began to attract real attention around 2003 when it started showing up in military hospitals during the Iraq War. Its claim to fame is that it was one of the first bacteria to be called "panresistant" because it is resistant to almost all antibiotics. Before the emergence of panresistant *A. baumannii*, the worst threat in terms of antibiotic resistance was another soil bacterium, *Pseudomonas aeruginosa*, which has long been known as an infectious disease problem in burn victims and cystic fibrosis patients. A number of other soil bacteria and bacteria normally found in or on the human body (such as MRSA, mentioned previously) also seem to be resistant to multiple antibiotics. Unfortunately, these ubiquitous bacteria have emerged as common causes of hospital-acquired infections.

A new understanding of antibiotic resistance is that the physiological state of a bacterium can be as important as its complement of resistance genes. Many bacteria can form **biofilms**, multilayer groupings of bacteria that are held together by a sticky polysaccharide matrix secreted by the bacteria. Biofilms are found in many places in nature, most notably in areas such as streams, where the fast flow of water makes it necessary for bacteria to resort to biofilm formation to stay in a particular site. In hospital patients, biofilms seem to form very readily on plastic or metal implants. Although we do not yet fully understand the reason, bacteria in these biofilms are significantly less susceptible to antibiotics and are therefore quite difficult to eliminate. All too frequently, patients with a biofilm-contaminated implant have to undergo additional surgery to remove the implant so that antibiotics can be used to eliminate any remaining bacteria. After the infection is cleared, a second implantation can be attempted.

Bioterrorism

No discussion of current infectious disease issues would be complete without mention of bioterrorism. Germ warfare—the use of infectious microorganisms as weapons—is an old idea that has, fortunately, never worked very well. The nature of germ warfare has changed in recent years. In the past, the purpose of germ warfare was to kill or incapacitate large numbers of soldiers. Recently, the aim of terrorists' actions has changed. Now, the goal is to frighten the general population (hence the name "bioterrorism"). A small number of deaths are sufficient to achieve this goal.

Among bacteria, *Bacillus anthracis,* the causative agent of **anthrax** disease, has been identified as a particularly useful weapon by bioterrorists. *B. anthracis*, as a spore-forming Gram-positive bacterium, is easier to store and "weaponize" than a more fragile organism, such as the Gram-negative *Yersinia pestis*, the causative agent of bubonic plague. In spore form, *B. anthracis* is also easier to handle than the highly contagious smallpox virus. The U.S. Army was worried enough about possible anthrax attacks to administer the anthrax vaccine to soldiers going to Iraq and Afghanistan. This sparked controversy because the efficacy and safety of the available anthrax vaccines were contentious issues at the time. Unfortunately, the anthrax attacks through the U.S. postal system in late 2001 only served to exacerbate the fear and solidify the realization that bioterrorism is a reality that we must now address.

An alternative bacterial choice of bioterrorists is *Clostridium botulinum*, another spore-forming bacterium that produces **botulinum neurotoxin**. Producing botulinum neurotoxin in your garage is inadvisable and can be extremely hazardous to your health, but this toxin is produced commercially (as Botox) for use in a variety of medical and cosmetic applications, ranging from correcting facial tics and strabismus (cross eyed) to eliminating wrinkles and preventing scarring from reconstructive surgery. Thus, it is conceivable that terrorists might hijack commercially produced Botox from factories that produce it. Whether emptying vials of toxin into a city's water supply would actually result in any deaths is not clear, as dilution and breakdown of the purified toxin protein in the environment will occur. It is, however, better to err on the side of caution. The most recent concern, though so far only theoretical, is that botulinum toxin might be deliberately introduced into milk, juice, or soft drinks during processing.

A New Respect for Prevention

Major changes that hold great promise for the future have been occurring in the approach to controlling infectious diseases. Traditionally, medical establishments in developed countries have opted for a treatment-based approach. Although vaccinations were given to prevent some diseases and doctors used antibiotics prophylactically to prevent others, such as postsurgical infections or infections in cancer chemotherapy patients, the most common approach to treating infectious diseases was to wait for an infected person to seek medical help before intervening in the disease process. This approach has been criticized for being expensive and for allowing diseases to gain a foothold in the body before action is taken—a delay that in some cases results in long-term damage to the patient, even if the treatment successfully eliminates the infecting bacterium from the body.

Treatment-based approaches have also become much less effective as increasingly resistant bacteria make it more difficult to choose the appropriate antibiotic treatment. To better combat this escalating problem, it is important to understand the reasons for the rise in antibiotic resistance, particularly in hospital settings. If a bacterial infection is not cleared immediately, sepsis can kill a patient in just a few hours. Thus, since waiting for proper diagnostics can be fatal to the patient, the physicians' responses have generally been to use more advanced, broad-spectrum antibiotics to treat all bacterial infections, regardless of whether they might be treatable with less expensive, narrow-spectrum antibiotics. Adding the diagnostic testing to the decision process also raises the overall cost of the clinical visit, something that not only delays treatment, but also is actively discouraged by health insurance companies. Physicians have been advised to use the frontline antibiotics first, but also to send samples to the microbiological laboratory for antibiotic resistance evaluation and then adjust the therapy if laboratory results indicate another, more appropriate treatment regimen. Nevertheless, the overall result is increased selective pressure on the bacteria to develop resistance against the frontline antibiotics.

A far preferable approach to controlling a disease is preventing it in the first place. This approach has been successful in ensuring the safety of food and water. Now, more and more public health officials, hospital managers, and executives of health management organizations (HMOs) are rediscovering that prevention is also far more effective—and far less expensive—than treatment after infection has occurred. Prevention is suddenly center stage again. But, for a preventive approach to work, it is first necessary to have extensive information about the **epidemiology** of disease (i.e., information about disease patterns, their geographic distribution, and determinants of health-related states). It is also necessary to have a large-scale networking infrastructure in place that can serve as an early warning system to detect signals indicating new disease trends. Led by the CDC and the WHO, a variety of such epidemiological **surveillance programs** have been implemented to monitor the appearance of new diseases, the increased incidence of existing diseases, and the occurrence of antibiotic-resistant bacteria. Indeed, the CDC website (http://www.cdc.gov/) is now an extensive portal for the latest information about various infectious diseases, including scientific and medical information about a disease and its causative agent, up-to-date disease trends, precautionary measures, and travel alerts.

The CDC has been monitoring a subset of particularly problematic infectious diseases for years, but the list of diseases covered had been far from exhaustive. Now, many more infectious diseases, such as infections with pathogenic *E. coli* and *Chlamydia*, have been placed on the list of reportable diseases. A problem the CDC has had to cope with is that reporting of diseases is voluntary on the part of state public health departments. Overworked and underfunded state health departments have sometimes, understandably, given reporting of diseases a low priority. The CDC and the **National Institutes of Health (NIH)** are fighting to alert government agencies to the importance of having consistently funded monitoring programs. Many of the recent pandemics, such as SARS, West Nile virus, Ebola, and various avian and swine flus, have lent urgency to these efforts.

Surveillance: An Early Warning System

An example of a CDC surveillance program that was established in 1995 is **Foodnet**, a program that tries to count all cases of foodborne disease, such as those caused by *Salmonella*, *E. coli* O157:H7, *Vibrio cholerae*, *Listeria*, and *Campylobacter,* in 10 selected states in the United States and then to estimate from these data the incidence of these diseases nationwide. Attempts are also being made in several areas to monitor antibiotic-resistant pathogens. Prior to the introduction of Foodnet, the CDC had abundant information about large outbreaks of foodborne disease, but had no idea how many isolated cases of foodborne disease occur, so it was difficult to track sources of contamination. Based on the information gathered so far by Foodnet, the outlook is not positive. Although contamination of foods by some pathogens, such as *E. coli* O157:H7, has declined, there have been little or no recent reductions for most other infections. Indeed, *Campylobacter* and *Vibrio* infections have actually increased in recent years.

Monitoring disease prevalence is only the first step. Next must come effective action to control the further spread of disease. Due to limited shelf life, most perishable products must be shipped immediately for distribution. As such, companies generally wait until the final step in food processing to test foods for microbiological safety. However, it can take days to weeks for microbiological test results to be obtained. This scenario allows shipments of contaminated food to leave processing plants and reach points of distribution before the results of the tests are known. There are encouraging signs that programs for prevention of foodborne diseases are beginning to be implemented. For example, the **Food and Drug Administration (FDA)** and the **United States Department of Agriculture (USDA)** have now in place **hazard analysis and critical control point (HACCP)** programs.

Based on risk assessment, HACCP programs monitor the food and food safety practices at control points along the food production chain where contamination is most likely to occur. This approach not only lessens the likelihood that contaminated foods will be shipped, but also identifies contamination problems or situations that might lead to contamination early so that they can be rectified. At first the food industry was leery of the HACCP approach, viewing it as a needless and potentially expensive government intrusion, but the food industry has now become more enthusiastic about HACCP programs after seeing how expensive and injurious to the reputation of a company a large recall of contaminated products can be. A good HACCP program not only protects the public from disease, but also protects the company from recalls and lawsuits. Although there is still grumbling about specifics, the HACCP concept seems to be catching on.

Making Hospitals Safe for Patients

Fears concerning the prevalence of increasingly antibiotic-resistant bacteria have led to a number of changes in the way hospitals handle infectious disease cases. At one time, a surgeon could infect numerous patients and never even be informed of such incidents, let alone held accountable, due to lack of communication between physicians who care for patients during the postsurgical period and the surgeons who performed the operation. On top of that, hospitals with an infection control program would often delegate the job of infection control officer to someone of lower administrative authority who could not take action against doctors whose postsurgical infection record was poor or whose drug-prescribing practices were open to criticism. Now, this job is being taken much more seriously and is usually held by an infectious disease physician. Communication lines are also being improved.

Managed health care organizations, alarmed at the costs associated with infections involving antibiotic-resistant bacteria, have started cracking down on physician abuse or overuse of antibiotics. Ironically, cost-cutting measures by the health care plan bean counters, who had pressured physicians earlier to reduce drastically the number of laboratory tests they ordered, actually contributed to the resistance problem by encouraging physicians to use the strongest drugs available regardless of need. Thus, although there is still a certain amount of confusion and sending of contradictory signals between managers and physicians, awareness of the consequences have spurred positive changes in diagnostic and antibiotic prescription practices.

A somewhat less successful effort, so far, has been the campaign within hospitals to persuade health care workers to wash their hands after each patient and to implement other supposedly standard precautions, such as changing gloves when moving from one patient to another. Because many outbreaks of hospital-acquired infections likely spread by contact with health care workers, preventing transmission by this route has become an important priority. Unfortunately, health care workers have gotten out of the habit of washing their hands and instead rely on antibiotics. The high-stress atmosphere of a modern hospital, in which fewer health care workers are expected to treat more and sicker patients, has made it difficult to perform hygienic procedures by the book.

Now that antibiotics are becoming less effective, hygienic practices have become more important than ever, yet there is still considerable disparity among hospitals regarding compliance. An article published in the *Wall Street Journal* (April 5, 2006) described some of the methods used by hospitals to increase hand hygiene among health care workers. The methods included educational programs, monitoring of activities, disciplinary action, and even dismissal for failure to comply. A more recent trend, the use of alcohol-based antimicrobial gels by health care workers, is starting to make a difference because these gels are more effective than soap and water, require only 15 to 30 seconds of application, and, unlike soaps, do not cause skin dryness.

And Now for Some Good News: You've Got a Bacterial Infection!

Who would have thought that a person could be happy to learn that he or she had a bacterial infection? Yet, this is exactly what has happened to people suffering from ulcers and some other chronic infections. The doctrine for decades has been that infectious diseases are acute diseases that develop rapidly and run their course quickly, whereas chronic diseases—diseases that last for long periods of time without resolving—are caused by an autoimmune response, a genetic disorder, or some environmental factor. Examples of chronic diseases are heart disease, Alzheimer's disease, and cancer. Now, one might ask the question: what if microbes caused these diseases and others like them? They might then be curable by antimicrobial agents or preventable by vaccines or other measures!

The discovery that many cases of liver cancer are caused by the hepatitis B virus made it possible to prevent this type of cancer by an antihepatitis B vaccine. Similarly, the discovery that most gastric and duodenal ulcers and some stomach cancers were caused by bacteria led to a revolution in the way patients with gastric diseases are now treated. More recently, cervical cancer was linked to the presence of human papilloma virus (HPV), and vaccination against HPV is now encouraged for women to prevent cervical cancer. Such examples have spawned a revolution that has led to the reinvestigation of virtually every chronic disease, from heart disease to schizophrenia, for possible microbial or viral origin.

The *Helicobacter pylori* Revolution

Not all is gloom and doom on the infectious disease front. Frustration over the inability of immunologists to find cures for autoimmune diseases or the inability of physiologists to find cures for heart disease has yielded to optimism as scientists begin to suspect that bacteria or other infectious agents may cause many of these diseases. To most people, taking lifelong medication that does not necessarily prevent the disease is not an acceptable "cure." Because microbiologists have by far the best track record for cures, the new rallying cry has become "Let's find the microbe that causes this intractable disease so we can cure it!"

The landmark that dramatically changed the way people think about chronic diseases was the discovery that most gastric inflammation and ulcers are caused by the Gram-negative bacterium *H. pylori*. This discovery led to a simple antibiotic combination therapy that cures gastritis and ulcers. Although some people have recurrences, the rate of recurrences is far lower than that for conventional treatments, which addressed the symptoms rather than the cause of the disease. Because having ulcers for a prolonged period increases the risk of developing gastric cancer, a particularly dangerous form of cancer, an effective treatment for ulcers should also help reduce the incidence of gastric cancer.

This discovery generated great enthusiasm among gastroenterologists, but when this information first came out, there was one major sector of the health care system that did not share in the celebration: the pharmaceutical companies. Ulcer medications, which had to be taken daily for life—costing patients thousands of dollars a year—were suddenly replaced by a single course of antibiotics that cost as little as $200 per treatment. Seeking to cut their losses, the pharmaceutical companies are now marketing their former prescription ulcer drugs as nonprescription heartburn medications.

The Aftermath

Getting the medical community to accept the idea that bacteria could cause ulcers took many years of acrimonious scientific debate, but once the idea was accepted, first by the research community and then by clinicians, it only took a short time for the world at large to understand that the implications of this discovery went far beyond ulcers. Suddenly, they recalled that if a disease is caused by a bacterium, then it could usually be cured with antibiotics, especially if diagnosed early enough. What followed was a veritable gold rush to find a bacterial cause for other diseases with unknown origins.

A partial list of the diseases currently being reexamined for possible bacterial origins is provided in Table 1-1. The scope of this list conveys better than anything else the boundless optimism that surged through the medical community once the implications of *H. pylori* as a cause of ulcers were fully appreciated. Table 1-1 indicates that some of these causal associations are well-established, whereas others are still controversial and may not pan out as a direct cause. Yet if even a few of these diseases become curable due to having bacterial origins, a treatment revolution will have occurred. More details about some of these associations will be given in subsequent chapters. It is striking how rapidly great skepticism (about *H. pylori* as a cause of ulcers) metamorphosed into great optimism about the likelihood of making further discoveries of similar magnitude.

Microbiota Shift Diseases

A category of bacterial disease that defies conventional classification consists of diseases that are not caused by a single bacterial pathogen, but rather by a shift in the composition of a bacterial population of some part of the human body. Although the natural microbial populations (**microbiota**, formerly called microflora) of the human body are usually protective, certain shifts in the composition of these populations can have pathological consequences. Diseases of this

Table 1-1. Some diseases currently suspected of being caused by bacteria

DISEASE	SUSPECTED MICROBE (STATUS OF DISEASE ASSOCIATION)
Gastric ulcers	*Helicobacter pylori* (well-established causal role)
Gastric cancer (some cases)	*Helicobacter pylori* (etiologically linked to gastric adenocarcinoma and lymphoma)
Periodontal disease	*Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum,* and other oral bacteria (established as etiologic agents)
Atherosclerosis (and vascular disease)	*Chlamydophila pneumoniae* (epidemiological and experimental evidence indicate as a possible risk factor; biological plausibility shown in animal models)
Low birth weight and preterm babies (some cases)	Bacterial vaginosis and shifts in vaginal bacterial populations (established association with increased risk)
Cerebral palsy (some cases)	Placental or intrauterine infection (possible risk factor in multifactorial condition, established association with preterm birth for which infection is a high risk factor)
Rheumatoid arthritis	Intestinal anaerobic bacteria, *Borrelia burgdorferi* (Lyme disease), mycoplasmas, viruses (possible triggering agents; strong correlation with autoimmunity triggered by periodontal or lung infections)
Crohn's disease	*Mycobacterium avium, Mycobacterium paratuberculosis,* or shifts in intestinal bacterial populations (strong experimental and epidemiological association; biological plausibility shown in animal models of disease)

type are referred to as **microbiota shift diseases**. In chapter 5, "The Normal Human Microbiota," examples of microbiota shift diseases are described in more detail, but for present purposes, one example should suffice: **bacterial vaginosis**.

Bacterial vaginosis is the term used to describe a shift in the vaginal microbiota from a predominantly Gram-positive population, dominated by *Lactobacillus* species, to a population of Gram-negative anaerobes. For a long time, this condition was not taken seriously by physicians because the only symptoms, if there were symptoms at all, were a sparse discharge, some discomfort, and in some women a fishy odor. Two papers in the *New England Journal of Medicine* in 1995 changed the status of bacterial vaginosis. One of these papers linked bacterial vaginosis with preterm births. This was an epidemiological association, not proof of a cause-and-effect relationship. The second paper described the result of an intervention study, in which antibiotics known to target Gram-negative anaerobes were administered to pregnant women with bacterial vaginosis, and the effect on the birth weight of the infant was determined. Antibiotic intervention that returned the vaginal microbiota to "normal" was associated with normal full-term births, whereas untreated women were significantly more likely to have preterm infants.

Soon after these first connections were made, bacterial vaginosis was linked to a higher risk of contracting HIV infections and other sexually transmitted diseases, just as chlamydial disease and gonorrhea had been shown previously. A major challenge for scientists trained in the analysis of diseases caused by a single species of microorganism is to learn how to deal technically and conceptually with polymicrobial diseases caused by shifts in bacterial populations consisting of hundreds of species. Undoubtedly, all of the species present are not equal contributors to the disease state, but the situation is far more complex than single-microbe infections.

A Brave New World of Pathogenesis Research

The *H. pylori* revolution captured the public imagination, but an even more important revolution has been the realization by research scientists that new molecular technologies are opening up a plethora of new opportunities to understand at the molecular level how infectious diseases develop. For several decades after the discovery of antibiotics, during a period in which a number of new vaccines were developed, it seemed sufficient to simply treat or prevent bacterial diseases. As long as antibiotics worked and vaccines were widely available, controlling bacterial infections at the practical level did not require in-depth information about the bacterium-host interaction. As physicians and scientists became concerned about increasing antibiotic resistance, there was a growing realization that a better understanding of the detailed interactions between the human body and the bacterial pathogen might lead to new treatment strategies.

Additionally, there was recognition that there are some diseases whose symptoms are caused by bacterially produced toxins that are not effectively treated by antibiotics. A good example is anthrax, a disease caused by *Bacillus anthracis*. The symptoms of this disease are caused by a protein toxin, produced and secreted as the bacteria multiply in the body. If the disease is diagnosed immediately and the right antibiotic is administered, the disease can be controlled. However, antibiotics do not inactivate the toxin, and if antibiotic therapy is delayed for even a few days, enough anthrax toxin will have been produced to cause death.

In the case of a disease called **septic shock** that develops when bacteria enter and proliferate in the bloodstream (sepsis), a nonprotein component of the Gram-negative bacterial cell surface, **lipopolysaccharide (LPS)** (see Box 1-4), acts as a toxin that leads to organ damage and death. Here too, antibiotics are only effective if they are administered very early in the infection before the bacteria lyse and release too much of this toxic material. Although anthrax is not a significant threat (other than as a potential bioweapon), septic shock continues to kill tens of thousands of people each year in the United States alone. As discussed in later chapters, new understanding has recently emerged about how the human body responds to LPS, as well as to nonprotein surface components of Gram-positive bacteria, such as **lipoteichoic acid (LTA)**. There is growing hope that this knowledge will make possible new and more effective therapies.

A different type of question is why different people respond differently to the same bacterium. In some cases, the human response can range all the way from a virtual lack of symptoms in some individuals to severe illness and death in others. Taking this issue one step further, prior exposure to one microbe can impact the nature and degree of response to subsequent exposure to another microbe. Indeed, it is now well-established that maternal exposure to pathogens or their components (e.g., LPS) modulates the immune competence and immune response of offspring in species ranging from insects to plants to birds and mammals. Clearly, it would be helpful to understand this range of reactions so that people who are most susceptible to an infectious agent could be quickly identified and given priority in treatment.

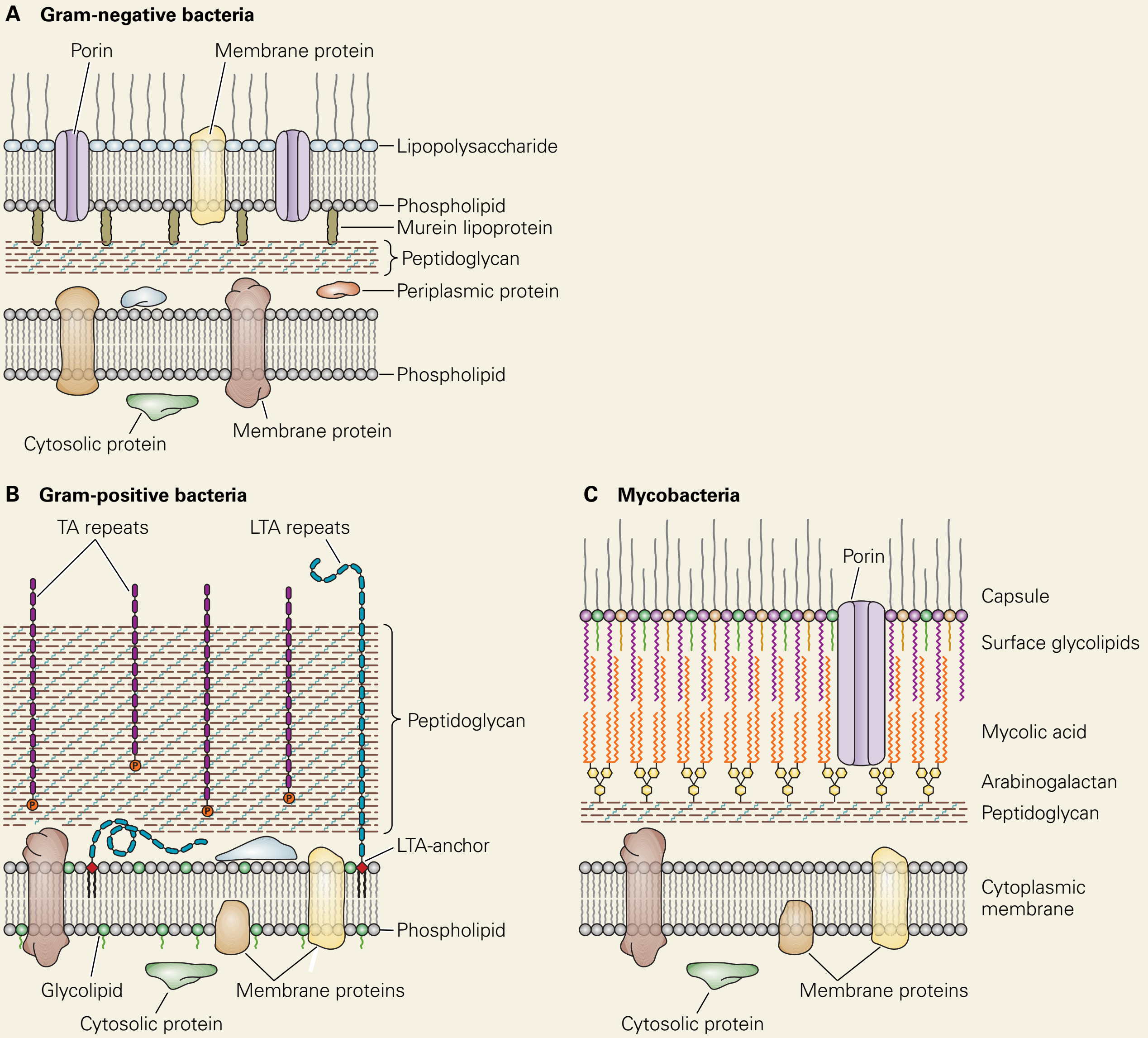
These and similar practical problems with controlling bacterial infections have driven a new interest in the interaction between bacteria and the human body at the molecular level. Fortunately, a cornucopia of new molecular tools and paradigms has become available that have made it possible to explore the host-pathogen interaction in a detailed way. It has even become more feasible to investigate infections that involve more than one species of bacteria or infections in which the bacterial pathogen acts in an area of the body, such as the mouth or small intestine, where there are many other bacteria that may influence the course of the disease.

Box 1-4.

A Brief Review of the Surfaces of Gram-Negative and Gram-Positive Bacteria—One Membrane versus Two

For convenience, bacteria are often divided into two main groups, Gram positive and Gram negative, based on their ability to retain a purple dye after being washed with organic solvent such as methanol or acetone (this procedure of coloring bacteria is called Gram staining). The LPS is located at the surface of Gram-negative cells and is composed of lipid A (composed of fatty acids and a disaccharide), the core oligosaccharide, and the repeating-unit O-antigen polysaccharide (panel A). The LPS forms the surface of the outer membrane, whose inner layer is composed of phospholipids. The thin peptidoglycan (also called murein) of Gram-negative species is located in the periplasm between the inner and outer membrane.

In contrast, the cell wall of Gram-positive species lacks LPS and an outer membrane (panel B). The thick peptidoglycan is composed of multiple, cross–linked layers. A substantial


A Gram-negative bacteria
Porin
Membrane protein
Lipopolysaccharide
Phospholipid
Murein lipoprotein
Peptidoglycan
Periplasmic protein
Phospholipid
Cytosolic protein
Membrane protein
B Gram-positive bacteria
TA repeats
LTA repeats
P
P
P
P
Peptidoglycan
LTA-anchor
Phospholipid
Glycolipid
Cytosolic protein
Membrane proteins
C Mycobacteria
Porin
Capsule
Surface glycolipids
Mycolic acid
Arabinogalactan
Peptidoglycan
Cytoplasmic membrane
Cytosolic protein
Membrane proteins

(*continued*)

Box 1-4.

A Brief Review of the Surfaces of Gram-Negative and Gram-Positive Bacteria—One Membrane versus Two (*continued*)

amount of the Gram-positive cell wall is made up of another anionic polymer called wall teichoic acid (WTA, or TA) and lipoteichoic acid (LTA), both of which contain repeat units of ribitol or glycerol phosphate linked to amino sugars, sometimes to amino acids, such as D-alanine, and sometimes to ternary amines, such as phosphorylcholine. TA is covalently bonded to the peptidoglycan, whereas LTA is linked to a lipid anchor in the cellular membrane.

Other bacteria have cell walls with compositions that are a variation on one of these two types plus a few different components. For instance, the cell wall of mycobacteria, including *Mycobacterium tuberculosis* (the causative agent of tuberculosis), is similar to Gram-negative bacteria, but mycobacteria have a thick outer membrane comprised of two leaflets: an inner leaflet composed of arabinogalactan and mycolic acid (hence the name "mycobacteria") and an outer leaflet composed of phosphoglycolipids (panel C). Further details can be found in *The Physiology and Biochemistry of Prokaryotes*, 4th Edition by David White, James Drummond, and Clay Fuqua (Oxford University Press, 2011).

It should be noted that the differences in bacterial surfaces and their components between Gram-positive and Gram-negative bacteria often dictate how they interact with their environments and, in particular, how they interact with their hosts. Importantly, the host immune system responds quite differently to these components. The properties conferred by these components necessitate that hosts have alternative strategies to recognize and combat Gram-negative versus Gram-positive pathogens. The presence of the outer membrane in Gram negatives also creates an extra challenge for the bacteria to export molecules on its surface and to secrete its toxins and other virulence factors into the external medium.

The New Age of Genomics

Tremendous breakthroughs in **DNA-sequencing technologies** and bioinformatics now allow scientists to rapidly sequence, analyze, and study entire bacterial genomes (also known as **genomics**). There are now more than 13,000 complete genome sequences for bacteria, about half of which are from medically relevant bacteria, and there are over 175,000 incomplete or ongoing bacterial sequencing projects. These numbers and the pace of discovery are staggering considering that only a handful of complete bacterial genomes were available in the year 2000. At one time, it would have made sense to provide a list of available genome sequences, but additions to this list are coming so fast that the best solution is to provide the address of a website that keeps track of genomes that have been or are being sequenced: https://gold.jgi.doe.gov/.

Once a **genome sequence** becomes available, scientists examine the **open reading frames (orfs)** (i.e., the putative genes) one by one to try to assign each gene a name and function. In some cases, this process of genome **annotation** is easy, because the gene and its expressed protein product have already been characterized. In other cases, tentative identification of a gene is made on the basis of similarities to known genes or proteins present in other organisms that have been deposited into public repositories for DNA and protein sequences—**DNA databases** and **protein databases**, respectively. These automated assignments are useful but should be treated with some degree of caution, as many are based on relatively poor sequence matches. The best way to approach DNA sequence data is to realize that the function of a gene based on its sequence similarity to known genes is only a hypothesis that needs to be confirmed by more rigorous testing. A sobering fact is that even in the case of well-studied bacteria, such as *E. coli* and *Salmonella*, at least one-third of the genes in their genomes have no similarity to any known genes. A challenging job for future scientists is to determine the biological role of these genes of unknown function.

The way in which DNA sequence information can reveal surprising things about an organism is illustrated by the genome sequence of *Borrelia burgdorferi*, the spirochete that causes Lyme disease. Scientists noted that no genes corresponding to the usual iron-containing proteins normally found in bacteria were present in the genome of this organism. This suggested a radical hypothesis: that *B. burgdorferi* copes with the problem of low iron concentrations in the mammalian host by not using iron at all. Instead, its proteins use other metals that are abundant in humans, such as manganese. Scientists who were trained in an era in which every article on iron utilization by bacteria started by describing that all bacteria require iron were startled by this suggestion. Biochemical analyses confirmed, however, that indeed *B. burgdorferi* apparently does live without the need for iron, thus solving one problem most other pathogens have to confront: how to obtain iron in a host whose iron sequestration mechanisms keep the supply of available iron very low. As can be seen from this example, genome sequences not only provide valuable insights into unique bacterial metabolic processes, but also are excellent hypothesis-generating tools for understanding virulence mechanisms.

Along with the availability of complete genome sequences has come new technology enabling the high-throughput sequencing of an organism's total RNA (**RNA-seq**, also called whole transcriptome shotgun sequencing). This technology has provided the means for scientists to measure the expression of thousands of genes in a single experiment. If the number of bacteria is high enough in a body site of a colonized or infected animal, RNA isolated from bacteria growing under these *in vivo* conditions can be obtained and analyzed by RNA-seq to assess the expression profile (i.e., the **transcriptome**) of different genes in the animal. Comparison of this expression profile with that obtained from bacteria grown outside of the host body has led, in turn, to the identification of genes that are only expressed during an infection and that might contribute to virulence in the host.

Another form of genomic analysis being used to detect and identify unknown pathogenic bacteria takes advantage of the fact that ribosomal RNA (rRNA) genes contain highly conserved regions of sequence separated by more variable regions. PCR primers that target conserved regions of the rRNA genes are used to amplify these genes from genomic DNA extracted from tissue suspected to contain an infectious organism. The PCR-amplified DNA is called an **amplicon**. Of course, if there are no bacteria present or if the level of bacterial DNA is too low, no PCR amplicon will be obtained; however, if an amplicon is obtained, its sequence can be determined and compared to the thousands of rRNA gene sequences now available in the DNA databases.

The variable regions of the 16S rRNA gene are particularly valuable in helping determine what known microbe is most similar to the one found in the diseased tissue. The next step is to establish whether the amplified DNA that comes from that organism is present in all cases in which there are similar symptoms of disease. The first unknown organism to be identified in this way was the bacterium that causes a rare intestinal disease called Whipple's disease (*Tropheryma whipplei*). Currently, this approach is being used in an attempt to identify bacterial pathogen(s) thought to be responsible for other diseases with unknown causes (i.e., etiology), such as bacterial vaginosis, atherosclerosis, and inflammatory bowel disease.

Insights into Pathogen Evolution

A current preoccupation of many infectious disease researchers interested in deciphering the root causes of pathogen evolution and the dynamics of epidemics is to combine epidemiological and evolutionary knowledge about pathogen virulence gleaned from genomics. Application of mathematical modeling is then used to gain better understanding of pathogen physiology, ecology, and disease transmission. In formulating these models, the two most common assumptions are that pathogens evolve in response to selective pressures placed on them by their environment, namely the host—and, as we are beginning to learn, also the external environment—and that virulence (i.e., the deleterious effects of the pathogen on its host) is directly proportional to its ability to be transmitted.

Recent studies along these lines have provided new insights into the potential effects of imperfect vaccines and the reemergence of certain diseases once thought eradicated (or at least well-controlled) through vaccination, such as whooping cough. It has been a generally accepted premise that infection with one strain of a bacterial pathogen will significantly reduce the susceptibility of the host to subsequent infections with other related bacterial strains due to the host's acquired immunity. However, these recent studies now indicate that the selective pressure of immunization may also be driving the evolution of pathogens like *Bordetella pertussis,* the bacterium responsible for whooping cough. Genomic sequence comparisons indicate that *Bordetella* strains, against which most of the current vaccines were designed and used extensively in developed countries, have now been replaced in the population by novel variant strains lacking key components previously recognized by the immune system to help clear the pathogen from the body. Experiments in mice further suggest that the existing vaccines are less protective against some of these new variants.

Modeling the Host-Pathogen Interaction in Experimental Animals

Studies of disease-causing bacteria growing under laboratory conditions need to be supplemented by studies in animal models. The most familiar type of infectious disease model is the laboratory rodent. Inbred strains of mice and rats are widely used as models for the infection process. The availability of animals with known genetic mutations in their defense systems has increased the utility of these rodent models. Numerous examples of the use of these models will be seen in later chapters of this book, including discussions of when it is appropriate to use animals in experimental schemes to study infectious diseases and the ethics of using vertebrate animals in experiments.

A new wrinkle on the animal model story is the increasing range of "animals" used, from the nematode *Caenorhabditis elegans* to the fruit fly *Drosophila melanogaster* and the zebra fish *Danio rerio*. Certainly, one motivation for using such models is the fact that the complex body of regulations and restrictions that has built up around the use of laboratory rodents and other warm-blooded animals is so complicated and expensive that only the best-funded laboratories can use them. However, more compelling reasons for the use of these new models are that so much is known about their genetics and that they are much more easily and rapidly genetically manipulated than mammals.

In the case of *C. elegans*, for example, the developmental origin and fate of every cell in the whole organism is known.

Drosophila has a long history of use as a model for insect and human genetics, and many characterized mutants are available. In fact, a type of receptor on human neutrophils that is important for responding to bacterial infections (Toll-like receptors) was first discovered in *Drosophila* (Toll receptors). The zebra fish is a newer infection model, but it also has some of the same attractive features as the nematode and fruit fly models (e.g., small size, easy maintenance, short generation time, and ease of genetic manipulation). Zebra fish have the added advantage that they have somewhat more advanced host defense systems than the nematode and fruit fly and are thus a better model for the human immune system, particularly with regard to aquatic pathogens.

Given the genetic distance between these animals and mammals, a certain degree of care must be used in choosing the experimental questions and interpreting the results. For example, although nematodes, fruit flies, and zebra fish have phagocytic cell defenses that exhibit some similarities to that of humans, the systems are not identical and are evolutionarily distant from mammals. For example, insects and worms lack adaptive immune responses as are found in humans and other mammals, and so cannot be used to study antibody responses and inflammation. They also lack many of the immune signaling components present during infection in mammals. While zebra fish are genetically tractable vertebrate models with complete adaptive immune responses in adults, only a few of the immune components have been functionally studied thus far. Indeed, recent studies have also revealed questions regarding the evolutionary conservation of some of the processing and activation of inflammation in zebra fish. Nonetheless, these simple models can be used to generate hypotheses that can later be tested in laboratory rodents or other animals and, in some cases, humans.

Correlation Studies

Another type of modeling that has been used for a long time in epidemiological studies but is relatively new to pathogenesis studies is the statistical analysis of not just microbial populations, but also human and animal populations. At present, this type of modeling is still rather unsophisticated and based on seeking correlations between traits of the organism and outcomes of disease. In other words, the model can be used to ask whether the production of a particular protein is associated in a statistically significant fashion with various aspects of the disease progression in humans. This approach has the advantage of being easy to do because one merely needs to apply preexisting statistical methods. There are, however, two rather serious problems with this approach.

First, this kind of "modeling" is not modeling in the sense that this term is used in physics or chemistry, in which principles are first expressed mathematically in a way that generates specific predictions about the outcome of an experiment. Instead, correlation studies are usually performed without any clear idea of a theoretical connection between the parameters being tested. As such, finding a correlation does not prove cause and effect. There is an urban legend that illustrates this. A gentleman in California happened to pull down a shade in his apartment just before the onset of a particularly severe earthquake and remained convinced for the rest of his life that pulling down the shade had helped to cause the earthquake.

A second problem is that the items to be checked for correlation are chosen by the researcher, and there may or may not be some theoretical underpinnings to the choice. These problems do not necessarily make the correlation studies inappropriate, but these issues do emphasize the need for scrutiny. If the approach is treated as one for potentially generating hypotheses rather than as a method that provides a proof of cause and effect, then the objections disappear. As more mathematicians, physicists, and bioinformatists are becoming interested in applying their tools to study infectious diseases, more sophisticated modeling approaches are beginning to emerge.

As important as new technologies have been, the most important advance has been a new appreciation for the importance of focusing not just on the properties of a bacterium in a test tube, but also on the myriad ways in which the bacterium interacts with its environment and stimulates responses from the human body. In this book, we will place great emphasis on this bacterium-host interaction. It will become clear very quickly that although considerable progress has been made, there is much to be learned and many opportunities for readers of this book to participate in future research in the area of bacterial pathogenesis.

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Questions

1. The number of human deaths is often used as a standard for ranking human diseases in terms of importance. What, if anything, is wrong with this classification scheme?
2. Infectious diseases have obvious deleterious effects on the infected individual. Are there other consequences that reach beyond the infected person to his or her family and to society as a whole?
3. The United States and most developed countries have long had medical communities that focus on therapy rather than prevention. Why is this the case and under what conditions might this emphasis be appropriate? Why are scientists arguing for a return to a prevention-based health care system?
4. In our classification of emerging or reemerging infectious diseases, we treated antibiotic-resistant bacteria and *E. coli* O157:H7 as new diseases. Make the case for and against considering a member of an established disease-causing species that acquires a new trait as a new disease entity. What is the significance of such changes in bacterial pathogens and our ability to treat them?
5. The bacteria that cause the diseases cholera and tuberculosis are much more infectious than the so-called "opportunists." Why then are these opportunists currently much more of a health concern in developed countries than cholera or tuberculosis?
6. Under what conditions—assuming no new epidemics—could infectious diseases suddenly move to the second or even first most common cause of deaths in the United States?
7. Do you think humans will ever win the battle against disease-causing bacteria? Why or why not? Is the use of warlike language to describe the relationship between humans and bacteria even accurate?
8. Microbiologists are fond of saying that only a tiny minority of bacteria causes disease. Are there reasons for thinking this might not be true?
9. In what sense are bacteria life-givers rather than life-takers? Is it possible that disease-causing bacteria might have a beneficial role in another context or even in their relationship with the human body?
10. There are many diseases that manifest in a variety of ways in different, apparently healthy individuals (e.g., a bacterium may cause a mild fever and malaise in one person, while causing life-threatening disease in another). What are some of the factors that may contribute to this phenomenon?

2

IN THIS CHAPTER

The Best Defense: Avoid, Reduce, and Prevent Exposure!

Barriers: Skin and Mucosal Membranes

- The Layers of Cells That Protect the Body
- Normal Microbiota of the Skin and Mucosa
- Defenses of the Skin
- Defenses of Mucosal Surfaces
- Special Defenses of the Gastrointestinal Tract
- Special Defenses of the Urogenital Tract
- Special Defenses of the Respiratory Tract

Immune Defenses of the Skin and Mucosa

Models for Studying Breaches of Barrier Defenses

Selected Readings

Questions

CHAPTER 2

Skin and Mucosa

THE FIRST LINES OF DEFENSE AGAINST BACTERIAL INFECTIONS

During medieval times, castles were built to serve not only as a residence for the feudal lord and his family and subjects, but also as a fortress to keep marauding enemies out. The most effective castles were designed by medieval engineers to last a long time, with high stone walls to withstand the onslaught of an enemy attack or prolonged siege. They were built in strategic locations surrounded by challenging terrain, such as on high ground with impassable moats or on top of steep cliffs. They were also poorly accessible, often with at least one side facing a waterfront, such as a river, lake, or ocean. Roads leading to the castle were usually twining and inclined to restrict access by wagons and hinder passage of large invading armies. Vigilant soldiers standing at the gates and archers placed along the top of the walls and in lookout towers guarded the castle against any would-be invaders that did approach the castle walls. And, if these barriers were still breached, the knights would mount fierce battles to protect the castle's inhabitants. Like these medieval castles, our bodies are built as nearly impregnable fortresses, with analogous defensive strategies that protect the body against siege and invasion by potential microbial adversaries. Clearly, an attacking army required considerable ingenuity to breach the castle's defenses. And, like the more successful medieval invader, the more successful invading bacteria (i.e., pathogens) have evolved clever virulence strategies to overcome those defenses of the body.

The Best Defense: Avoid, Reduce, and Prevent Exposure!

How is it possible that researchers and health care workers, who on a daily basis work with or are exposed to highly pathogenic microorganisms, do not get sick that often? This is because they have learned that the best line of defense is preventing exposure to a pathogen in the first place. In a research or health care setting, this can be achieved by avoiding direct contact through the use of gloves, protective clothing, and eyewear; disinfection of instruments and surface areas; and proper ventilation facilities. In the event that exposure does occur, other effective strategies come into play for preventing the pathogen from gaining entry into the body and establishing an infection. Of course, it helps to know the identity and properties of the pathogen that can be targeted for specific neutralization and also to bear in mind that any potential pathogen must always be treated with the utmost respect.

Reducing the number of bacteria that the body comes into contact with is a highly effective strategy to limit the risk of colonization and infection. There are many agents, natural (e.g., antibiotics, alcohols, and natural acids such as vinegar or citrus juice) or artificial (e.g., organic chemicals, drugs, and detergents), that can be used to kill or slow down the growth of microbes. Some of these agents can only be used externally on inanimate objects (e.g., disinfectants, sanitizers) or body surfaces (e.g., antiseptics, germicides) due to their harsh or toxic properties, but others are safe to ingest or inject in appropriate amounts (e.g., antibiotics, other antimicrobials). Personal hygiene through the use of handwashing with soap and water, as well as application of alcohol-based antiseptic gels, can also achieve the purpose of limiting exposure risks. We will revisit this topic later in chapter 15, when we discuss antimicrobial compounds.

Unfortunately, even with extra precautions and containment measures to reduce contact, accidental exposure can still occur through ingestion, inhalation, or direct contact with mucosal surfaces, such as the eyes or nose or through injury with sharp objects, needles, or abrasives. When this happens, it is comforting to know that the body has a number of impressive defense strategies already in place that make establishing an infection extremely difficult for the pathogen. The first of these strategies is a nearly impregnable physical and biochemical barrier that divides the external environment from the interior of the body. The second is the body's ability to mount a massive counteroffensive against any invading microbe through the ever-vigilant immune system. This chapter and the ones that follow are intended to provide an overview of the defenses of the human body that limit colonization and prevent infection. We will begin the discussion of these host defenses in this chapter with the physical and biochemical surface barriers and will continue in chapters 3 and 4 with discussion of the internal defenses and protection provided by the immune system. Finally, as covered later in chapter 17, "Vaccination: an Underappreciated Component of the Modern Medical Armamentarium," we will discuss how it is possible to further bolster this amazing defense system through vaccination and other therapeutic strategies.

Barriers: Skin and Mucosal Membranes

The skin and mucosal membranes of the human body are not simply inert physical barriers that keep good things in and bad things out. They comprise the body's largest organ, which has a complex array of activities and functions that are only now beginning to be fully appreciated. As humans and other life-forms evolved, they were forced to contend in a bacteria-dominated world in which bacteria tended to view them as a free lunch. Survival meant developing defenses to keep these bacteria at bay. As such, these fortress-like defenses are extremely effective against bacterial incursions, preventing most bacteria from entering human tissues and the bloodstream and instead relegating them to the surface of the skin or confining them to certain closely guarded mucosal areas of the human body, such as the **oronasopharyngeal** (mouth, nose, and pharynx/throat), gastrointestinal, and urogenital tracts. Fortunately for us, only a tiny minority of bacteria is able to bypass these defenses and cause disease. To understand the mechanisms by which pathogenic bacteria cause disease, it is first essential to know what obstacles those few bacteria that do cause disease must overcome.

The Layers of Cells That Protect the Body

Epithelia are the layers of cells covering all of the external and internal surfaces of the body that are exposed to the external environment. These epithelial cell layers are an important initial defense against potential pathogens. The epithelial cells found in different body sites differ considerably in their properties, but they have some features in common.

Skin consists of a lining of living cells composed of two layers, the **epidermis** (outer layer) and the **dermis** (inner layer) (Figure 2-1). Cells of the dermis are continuously replaced as they die and become a tough, dry outer layer called the epidermis. The epithelia that line the respiratory, intestinal, and urogenital tracts are the **mucosal epithelia** and consist of tightly packed cells that are attached to each other by protein structures called **tight adherens junctions** and **desmosomes** (Figure 2-2). The tight binding of epithelial cells to each other prevents bacteria from transiting an epithelial layer. To get through the epithelial layer, bacteria must either take advantage of wounds or be capable of invading epithelial cells, passing between or through them to get to the underlying tissue.

In contrast, the cells that line the surfaces of the interior of the body (the **endothelium**), such as blood vessels or lymphatic vessels, are not tightly bound to each other in order to allow the cells of the immune defense system to move freely from blood to tissues. Unfortunately, this feature also allows bacteria to move into and out of blood and lymphatic vessels by moving between the endothelial cells. Thus, once bacteria gain entry into the body at one site, it is possible for them to readily gain access to other parts of the body. Because of this vulnerability, it is imperative that the epithelia of skin and mucosal surfaces function as barriers against foreign invaders.

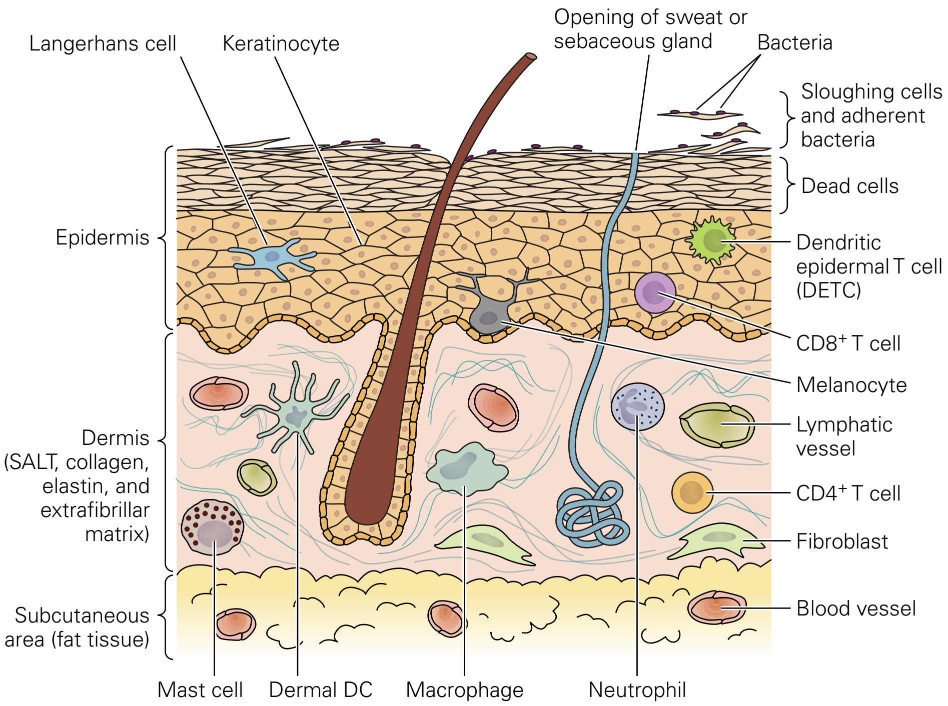

Langerhans cell
Keratinocyte
Opening of sweat or sebaceous gland
Bacteria
Sloughing cells and adherent bacteria
Dead cells
Epidermis
Dendritic epidermal T cell (DETC)
CD8⁺ T cell
Melanocyte
Lymphatic vessel
Dermis (SALT, collagen, elastin, and extrafibrillar matrix)
CD4⁺ T cell
Fibroblast
Subcutaneous area (fat tissue)
Blood vessel
Mast cell
Dermal DC
Macrophage
Neutrophil

Figure 2-1. Structure of the skin. The thick epidermis (outer layer, top) consists of keratinized, stratified squamous cells, the top layers of which are dead and continuously shed. When these cells are sloughed, they carry adhering bacteria with them. The inner layer is the dermis (middle). Sebaceous (fat) glands, sweat glands, and hair follicles, which produce antibacterial substances, provide openings in the skin through which pathogenic bacteria occasionally enter. The skin also has an adaptive defense system, the skin-associated lymphoid tissue (SALT). Langerhans cells serve as phagocytic cells in this system.

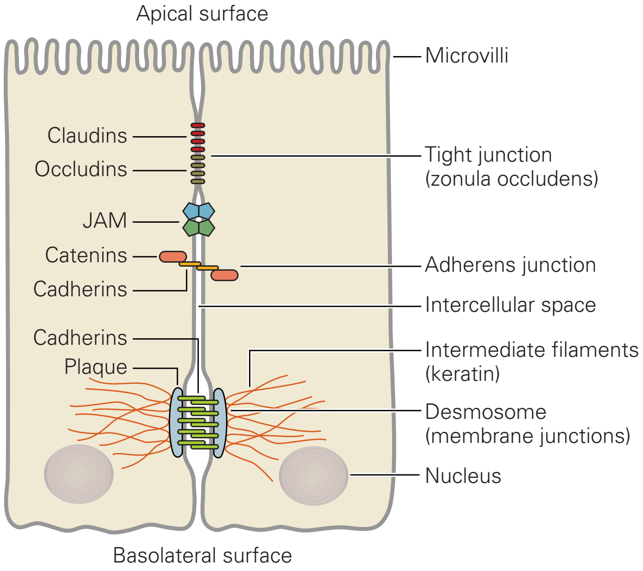

Apical surface
Microvilli
Claudins
Occludins
Tight junction (zonula occludens)
JAM
Catenins
Cadherins
Adherens junction
Intercellular space
Cadherins
Plaque
Intermediate filaments (keratin)
Desmosome (membrane junctions)
Nucleus
Basolateral surface

Figure 2-2. Intestinal epithelial cells showing tight, junctional, and adherens junctions, and desmosomes. JAM, junctional adhesion molecules.

The membrane surface of an epithelial cell that faces toward the interior tissues of the body and is attached to other cells or to connective tissues (**basolateral surface**) has a different protein composition from the membrane surface that faces outward (**apical surface**). Cells with this asymmetrical surface property are said to be **polarized cells**. A feature of epithelial cells is that they are attached to a thin sheet of connective tissue called the basement membrane (**basal lamina**). The basal lamina covers regions of **loose connective tissue** (Figure 2-3), comprised of **extracellular matrix (ECM)** secreted by elongated fibroblast cells. The ECM composition varies with tissue type, but primarily contains a network of interlocking gels of polysaccharides called **glycosaminoglycans** (such as chondroitin sulfate, hyaluronan, keratan sulfate, and heparan sulfate) attached to fibrous **collagens,** the most abundant protein type in the ECM. Collagens bind to adhesion glycoproteins called **fibronectins,** which in turn bind collagens to transmembrane cell surface proteins called **integrins** that mediate cell-cell and cell-ECM interactions and cytoskeletal responses. Other ECM proteins called **laminins** bind to collagens and other ECM components to form fibrous networks that resist tensile forces in the basal lamina. There are numerous examples of pathogenic bacteria that attach to components of the ECM and manipulate or mimic ECM components during the course of infection.

Epithelial cells in different body sites vary in shape, size, and number of layers (Figure 2-3), as well as in

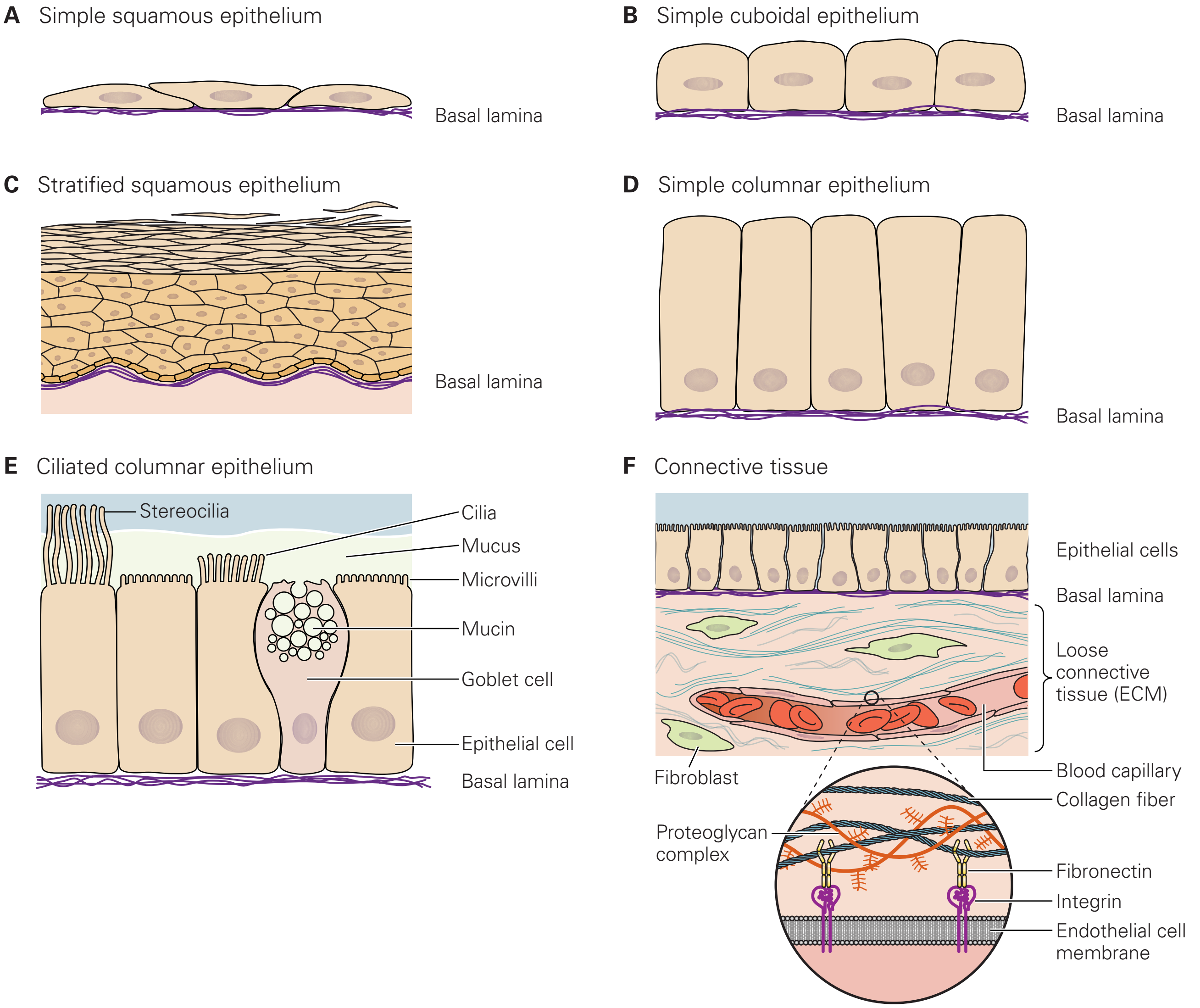

A Simple squamous epithelium
Basal lamina
B Simple cuboidal epithelium
Basal lamina
C Stratified squamous epithelium
Basal lamina
D Simple columnar epithelium
Basal lamina
E Ciliated columnar epithelium
Stereocilia
Cilia
Mucus
Microvilli
Mucin
Goblet cell
Epithelial cell
Basal lamina
F Connective tissue
Epithelial cells
Basal lamina
Loose connective tissue (ECM)
Fibroblast
Blood capillary
Collagen fiber
Proteoglycan complex
Fibronectin
Integrin
Endothelial cell membrane

Figure 2-3. Different types of epithelial cells and their relationships to underlying tissue. Shown are (A) simple squamous epithelium; (B) simple cuboidal epithelium; (C) stratified squamous epithelium (upper layers of cells are dead, typical of skin); (D) simple columnar epithelium; (E) ciliated columnar epithelium showing goblet cells, which secrete mucus (mucin); and (F) typical structure of connective tissue under an epithelial cell layer. Panel F modified from Cooper GM, Hausman RE. 2007. *The Cell—A Molecular Approach*, 4th ed. ASM Press, Washington, DC.

functional properties. Epithelial layers that cover surfaces where absorption or secretion is taking place (e.g., in the intestinal tract) usually consist of a single layer of epithelial cells (**simple epithelium**). Other surfaces, such as the female cervix or the skin, are composed of many layers of epithelial cells (**stratified epithelium**). Some have a flattened shape (**squamous epithelium**) and form the lining of cavities (such as the mouth, heart, and lungs) and the outer layers of the skin. Some are cube-shaped (**cuboidal epithelium**) and form the lining of kidney tubules and gland ducts and constitute the germinal epithelium that develops into egg and sperm cells. Others are tall and thin (**columnar epithelium**) and form the lining of the stomach and intestine.

Most of the surfaces that are exposed directly to the environment (e.g., the skin and mouth) are covered by stratified epithelia, whereas simple epithelia are found in internal areas, such as the intestinal tract or the lungs. Simple epithelia are more vulnerable to bacterial invasion than stratified epithelia because invading bacteria only have to pass through one layer of cells to gain access to the tissue underneath. We will use the terms mucosa epithelia, epithelial cells, mucosal layer, or mucosal cells to denote the simple epithelia of these internal areas.

Epithelia are protected by an array of innate and adaptive defenses. Some of these defenses are listed in Table 2-1 for the skin and Table 2-2 for mucosal surfaces. Other defenses, listed in Table 2-3, are more specific to certain areas of the body, such as the eyes and the respiratory, gastrointestinal, and urogenital tracts. For example, tears contain an enzyme (**lysozyme**) that degrades bacterial cell walls. Tears also provide a washing action that removes particles and bacteria from the eyes. Entry to the respiratory tract is protected by **mucus** and by specialized ciliated cells that propel bacteria-laden blobs of mucus out of the lungs. The urinary tract epithelium is protected by a sphincter at the end of the urethra, the tube that leads up to the bladder. This barrier makes it difficult for bacteria to enter the tube that leads to the bladder. Also, the washing action of urine during urination flushes out any bacteria that may have gained access to the bladder and urethra.

Normal Microbiota of the Skin and Mucosa

The defenses of the skin do not completely prevent bacterial growth, as is evident from the fact that there are some bacteria capable of colonizing skin and mucosal surfaces. Immediately after birth, a wide range of microbes colonize humans, particularly on the skin and in the oronasopharyngeal, gastrointestinal, and urogenital tracts. The members of a bacterial population that are found residing at a partic-

Table 2-1. Defenses of the skin

DEFENSE	FUNCTION
Dry, acidic (pH ~5) environment	Prevents growth of many bacteria
Cooler than internal body <37°C (typically 34–35°C)	Slows growth of bacteria that prefer optimal internal body temperature of ~37°C
Dead, keratinized cells	Hard to degrade and penetrate, discourages colonization
Sloughing of surface cells (desquamation)	Removes bacteria that adhere to surface cells
Oily secretion (sebum) containing toxic lipids, lysozyme, and defensins	Protects hair follicles, sweat glands, and sebaceous glands by preventing bacterial growth or by killing bacteria
Normal microbiota (mostly Gram-positive bacteria)	Competes with pathogens for nutrients, colonization sites
Skin-associated lymphoid tissue (SALT) underlying immune cells comprised of phagocytic cells (Langerhans cells), T cells, and B cells	Combat bacteria that manage to reach the dermis and tissue below it by initiating protective immune responses
Tight junctions between epithelial cells	Prevent bacteria from gaining access to internal body tissue

Table 2-2. Defenses of mucosal surfaces

DEFENSE	CHARACTERISTICS	PROTECTANTS	FUNCTION
Mucus (mucin)	Glycoprotein matrix	Lysozyme	Digests peptidoglycan, lyses growing bacteria
		sIgA antibodies	Bind and coat bacterial surfaces and mucin, traps bacteria in mucus that is sloughed off
		Lactoferrin	Sequesters iron, prevents growth of bacteria
		Phospholipase A2	Digests bacterial cytoplasmic membrane
Defensins	Small cysteine-rich cationic peptides of 18–45 amino acid residues	Antimicrobial cationic peptides, such as: α-defensins β-defensins	Disrupts bacterial cell integrity by inserting into bacterial membranes and forming pores, toxic for many bacteria
Secreted antibodies	Glycosylated protein complexes	sIgA	Bind and coat bacterial surfaces and mucin, traps bacteria in mucus that is sloughed off
Mucosa-associated lymphoid tissue (MALT)	Immune cells underlying mucosal surfaces or extruded between epithelial cells	Th cells	Recognize antigen bound APCs and regulate immune responses
		B cells	Produce IgA
		M cells	Engulf and transport bacterial cells from lumen across epithelial cell layer and deliver to DCs and macrophages
		Macrophages, DCs	Engulf and kill bacterial cells and present antigens to Th cells

Table 2-3. Special defenses of specific sites

SITE	DEFENSE
Eye	Tears (washing action and antibacterial substances; surface of eyeball)
Airway entrance	Mucus traps bacteria; ciliated epithelial cells propel bacteria-laden mucin blobs out of airway
Stomach	Acidic environment
Small intestine	Rapid flow of contents; sloughing of epithelial cells
Colon	Resident microbiota
Vaginal tract	Resident microbiota; sloughing of epithelial cells; flow of vaginal fluid
Bladder	Sphincter keeps bacteria out of urethra; flushing action of urine washes bacteria out of bladder

ular body site without causing disease are called the **resident (or commensal) microbiota** of that site.

The skin microbiota, consisting primarily of the Gram-positive bacteria *Staphylococcus epidermidis* and *Propionibacterium acnes*, help protect against pathogenic bacteria by occupying sites that might be colonized by pathogenic bacteria. They also compete with incoming pathogens for essential nutrients. Some resident bacteria also produce antagonistic bactericidal compounds (e.g., pore-forming toxins such as **bacteriocins** or growth inhibitors, which target other bacteria). The commensal microbiota does not completely prevent colonization of skin by potential pathogens, but hampers it enough that colonization by pathogenic bacteria is usually transient.

In the case of *P. acnes*, this anaerobic bacterium colonizes **sebaceous (fat) glands** and digests the oily **sebum**, composed of triglycerides, waxy esters, squalene, and free fatty acids, to help generate a low pH environment that is protective against other bacteria. Sebum production and secretion by sebaceous glands is increased by testosterone. During puberty, testosterone levels increase, particularly in males, and cause overgrowth of *P. acnes* in response to the abundance of sebum production. Air-oxidized sebum plugs the hair follicle or gland duct and gives rise to a clogged follicle (called a **comedo**) that is open (also known as a blackhead) (Figure 2-4). Dried sebum causes dead skin cells to adhere at the opening of the fat gland, which gives rise to a comedo that is closed and accumulates sebum (also known as a whitehead). The resulting anaerobic environment further enhances bacterial growth, causing infection and inflammation that manifests as acne (i.e., pimples or zits), or in severe cases as cysts or boils. Treatment with antibiotics and

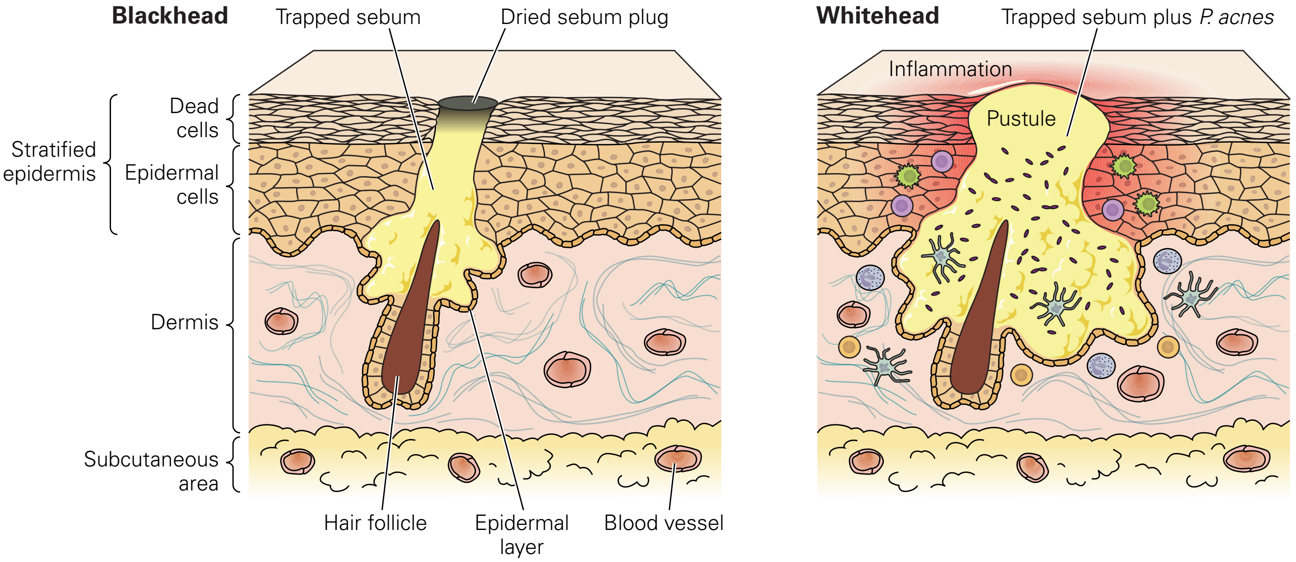


Figure 2-4. Skin infection with the commensal bacteria. Overgrowth of the commensal bacterium *Propionibacterium acnes* can lead to formation of a comedo that is open but plugged with air-oxidized sebum (blackhead) or closed by sebum-glued skin cells (whitehead). Inflammation of the comedo leads to acne (pimples).

Box 2-1.

Notable Breaches in Host Defenses that Changed the Course of U.S. History

Four presidents of the United States were assassinated during their presidencies: Abraham Lincoln (16th president: 1861–1865), James A. Garfield (20th president: 1881), William McKinley, Jr. (25th president: 1897–1901), and John F. Kennedy (35th president: 1961–1963). Kennedy died shortly after being shot, Lincoln died about nine hours afterward, and McKinley survived for about a week before dying, whereas Garfield lasted for 11 weeks before succumbing. Lincoln and Kennedy clearly died from complications due to the damage from the bullets' impact. In contrast, Garfield and McKinley did not die from the impact of the bullets that hit them, but instead they succumbed to the subsequent infections resulting from the wounds. In both cases, the lethal infections were caused by bacteria introduced by the physicians trying to remove the bullets lodged in their bodies.

In Garfield's case, one bullet grazed his shoulder and another hit his back, barely missing his spine before lodging in his pancreas, where doctors could not find it. Garfield was shot on July 2, 1881, and although his condition fluctuated with apparent signs of recovery alternating with fevers from infection, his illness worsened over the summer. Blood poisoning (sepsis) eventually took hold, and pus-filled abscesses formed all over his body. He finally died from uncontrolled septicemia (bacteria in bloodstream) and a ruptured splenic aneurysm on September 19, 1881. Most historians and medical experts are convinced that Garfield might have lived had the physicians of his time believed in aseptic technique while probing for the bullet, instead of using unwashed fingers and instruments.

Twenty years later, on September 14, 1901, McKinley died from gangrene resulting from bullet wounds received on September 6. Again, one bullet grazed McKinley while the other entered his abdomen and could not be found. Initially, he seemed to be on the mend, but his condition deteriorated rapidly on September 13 and he died early the next day. Although medical practices and precautions against infections were much improved by 1901, autopsy revealed that the bullet had penetrated the stomach, colon, kidney, and peritoneum along its way. These breaches to the normal epithelial barriers introduced the contents from the stomach and colon into the body's tissues and the bloodstream, leading to the subsequent sepsis and death.

Sources:

Leech M. 1959. *In the Days of McKinley*. Harper and Brothers, New York, NY.

Peskin A. 1978. *Garfield: A Biography*. The Kent State University Press, Kent, OH.

benzoyl peroxides found in most acne medications work against *P. acnes*. Washing with warm water and soap can also help reduce acne by keeping the pores open and free of dried sebum.

Although most mucosal surfaces are protected by normal resident microbiota (exceptions being the uterus and upper female genital tract and the urinary tract), the species composition of the microbiota found at different parts of the body varies from one site to another. Nonetheless, all have in common the predominance of Gram-positive resident bacteria. Shifts in these populations can be pathological, as is seen from diseases such as periodontal disease and bacterial vaginosis. The large intestine (colon) harbors an abundant and rich assortment of normal microbiota, the majority (97%) of which are anaerobes or facultative anaerobes. Many of these bacteria use carbohydrates and fats that are not digested by the stomach or absorbed by the small intestine. In return, some resident microbes provide a beneficial function to the host by synthesizing and secreting vitamins (e.g., vitamin K, vitamin B_{12}, and other B vitamins) and other nutrients that the intestine can absorb. Recent experimental evidence indicates that indigenous bacteria play a crucial inducive role in gut and immune development during early postnatal life. They stimulate development of certain tissues, in particular the caecum and Peyer's patches, the latter of which stimulate production of cross-reactive antibodies that prevent infection by related bacteria that may be pathogens (more on this later).

Members of the skin microbiota normally do not cause human infections unless they are introduced into the body by abrasions, catheters, or surgery. *Staphylococcus epidermidis*, a common skin bacterium, has been implicated in postsurgical and catheter-related infections. (*S. epidermidis* was the bacterial villain in the surgeon-transmitted infections described in Box 2-1.) Relatively nonpathogenic bacteria like *S. epidermidis* would normally be rapidly killed by the defenses of the bloodstream, but if they can reach an area that is somewhat protected from host defenses, such as the plastic surface of a heart valve implant, they can grow into surface-attached biofilms and produce quite serious infections. Catheters can provide skin-associated bacteria with a conduit into the bloodstream, thus bypassing the defenses of the epidermis and dermis. Catheter-associated infections

Box 2-2.
Handwashing Past and Present: A Lesson in Learning and Forgetting

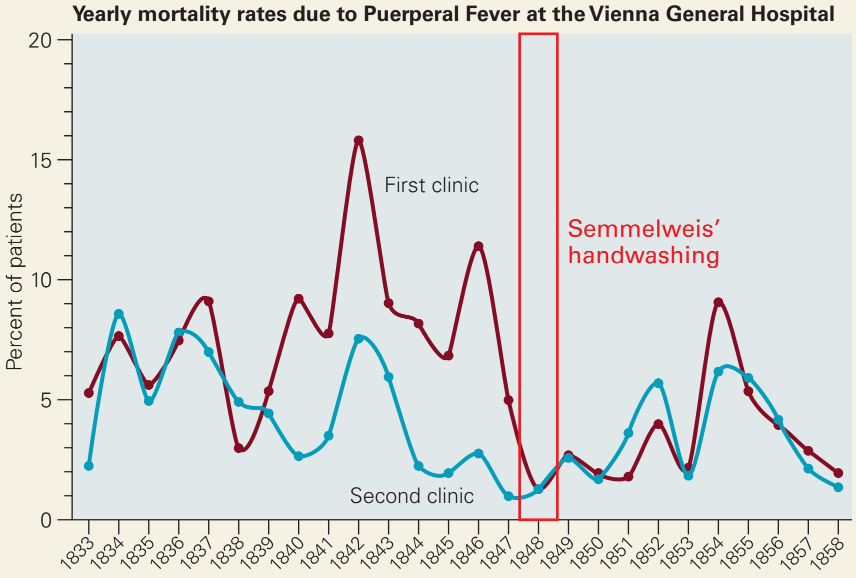
The idea that physicians and nurses should wash their hands before treating a new patient is actually not a recent innovation. Ignaz Semmelweis, the man credited with making handwashing a standard part of medical practice, lived and practiced medicine in the mid-1800s. Although he was not the first physician to make the connection between contaminated hands and the spread of disease by physicians to their patients, he was the first to prove that proper disinfection of hands could dramatically reduce hospital-acquired infections.

Semmelweis had noted that two maternity wards in the Vienna Lying-in Hospital had very different mortality rates. In one, the death rate due to puerperal "childbed" fever (a common cause of death in women of the period) was over 10%, whereas in the second ward it was less than 3%. This fact was well known to women entering the hospital, who considered assignment to the first ward to be a virtual death sentence. Both wards were equally crowded, with three patients sharing each bed and the sick mixed indiscriminately with the well. Both wards contained women of similar socioeconomic status. The only difference between the two clinics was that the first clinic was used for teaching medical students, who also were dissecting cadavers in between delivering babies, and the second was used for teaching midwives, who were not exposed to potential disease-carrying cadavers.

Semmelweis deduced that the medical students were transmitting childbed fever (which we now know is caused most frequently by the bacterium *Streptococcus pyogenes*) to their patients because they failed to cleanse their hands properly. In 1846, he instituted a policy requiring that all midwives and medical students wash their hands with a chlorinated lime solution before examining patients. The mortality rate in both wards promptly dropped to 1% (red box in figure). Unfortunately, Semmelweis' colleagues and detractors did not believe him and refused to follow his recommendations. His discovery remained controversial for many years, and it was only in the early 1900s that handwashing was universally accepted as an essential medical practice.

Today, proper disinfection of hands is one of the most basic and firmly entrenched of clinical procedures, especially for surgeons. Nonetheless, the advent of antibiotics and the consequent decrease in deaths due to hospital-acquired infections has led some surgeons to neglect this important practice. More recently, a surgeon in a large northeastern U.S. hospital, who started bypassing the rigorous surgical scrub procedure because he was troubled by dermatitis on his hands, provided a particularly dramatic example of this. The surgeon trusted the two pairs of surgical gloves, which were commonly worn during operations. But tiny holes in gloves can be made by contact with sharp objects or bone fragments. Also, the surgeon was using mineral oil to ease the irritation to his hands, and mineral oil undermines the integrity of surgical gloves. During the course of his duties, this physician managed to contaminate heart valve implants in a number of patients with *Staphylococcus epidermidis* before he was identified as the source of the outbreak.

S. epidermidis is commonly found as part of the resident microbiota of the skin, where it is not normally pathogenic, but it can cause infections if introduced into the body through wounds. Infections of heart valve implants usually cannot be



treated effectively with a simple course of antibiotics, not only because of the high resistance level of *S. epidermidis* strains, but also because of the formation of bacterial biofilms that are more resistant than individual bacteria to antibiotics. Thus, the patients with the infected valves had to endure a second operation to remove and replace the infected valves, not to mention additional damage to the heart due to the infection.

As is evident from the date on the reference cited at the end of this box, this case occurred in the 1980s. Does this mean that such cases have ceased to occur? Not at all! This case was used because it is a classic example of the handwashing problem, but there have been many other cases since. The difference between the 1980s and the 21st century is that the surgeon in this case would probably have been identified today before he infected so many people because infectious disease surveillance systems in hospitals have improved dramatically. But the attitude and behavior that sparked this episode do still sometimes occur in hospitals.

The silver lining in this particularly black cloud is that accountants for health insurance agencies have figured out how much the lack of handwashing and improper use of gloves are costing them, and they are mounting increasingly vigorous campaigns in favor of handwashing and against health care workers who ignore these simple but effective precautions. In fact, relatives of hospital patients are being urged to question unhygienic practices they witness. The lawyers are circling. Together these trends will continue to increase the safety of hospitals.

Sources:

Semmelweis I. 1983. *Etiology, Concept and Prophylaxis of Childbed Fever. Series: History of Science and Medicine (Book 2).* (Codell Carter K, translator.) University of Wisconsin Press, Madison, WI.

Carter KC, Carter B. 2005. *Childbed Fever: A Scientific Biography of Ignaz Semmelweis.* Transaction Publishers, Piscataway, NJ.

Boyce JM, Potter-Bynoe G, Opal SM, Dziobek L, Medeiros AA. 1990. A common-source outbreak of *Staphylococcus epidermidis* infections among patients undergoing cardiac surgery. *J Infect Dis* **161:**493–499 .

have become a serious enough problem in hospitals that catheter companies are developing plastic catheters that are impregnated with antibacterial compounds.

Surgical wound infections and catheter-associated infections caused by skin bacteria, especially *S. epidermidis,* have become an ever more prevalent problem due to the fact that many of these skin bacteria are now resistant to most available antibiotics. How has this happened? It is clear that at least some antibiotics are exuded in sweat. Also, ointments containing antibiotics are widely used in the treatment of skin conditions such as acne and rosacea (unnaturally red skin) over the course of months or years. Thus, it is not surprising that skin bacteria like *S. epidermidis* have become increasingly resistant to a variety of antibiotics. Moreover, growth of bacteria in biofilms increases any antibiotic resistance that already exists. Bacteria-containing biofilms will be discussed in a later chapter.

Since transient colonization with pathogens can occur and since even normally harmless skin bacteria can cause infections under certain conditions, handwashing and disinfection of hands adds yet another barrier to infection, and reduces transmission to other people with whom one may come into contact. In the mid-1800s, Ignaz Semmelweis first introduced the concept of handwashing and cleanliness in maternity wards (see Box 2-2). The low-tech, but very effective, protective barrier provided by handwashing and the frequent changing of gloves has probably been a key contributing factor toward the good safety record of research scientists. However, despite strong and convincing experimental data and persistent promotion of good hygiene policies by health care officials, compliance of health care workers with the recommended hygiene practices is still low, with rates of compliance sometimes lower than 50%. An added source of concern is the strong correlation between life-threatening nosocomial infections and the wearing of long or artificial fingernails, despite scrubbing practices. For example, from 1997 to 1998 the death of 16 babies in the neonatal ICU at a hospital in Oklahoma City was linked to a particular strain of *P. aeruginosa* found under the nails of three nurses.

To make matters worse, an alarming number of recent infectious outbreaks have been attributed to hospital-acquired pathogens (such as *Klebsiella pneumoniae, Pseudomonas aeruginosa, Candida albicans,* methicillin-resistant *Staphylococcus aureus* (MRSA), and *Serratia marcescens*) that also are resistant to multiple antibiotics. We will cover this serious problem in much more detail in chapters 15 and 16, when we discuss the topics of antimicrobial compounds and how bacteria become resistant to antibiotics.

Defenses of the Skin

Bacteria are unable to penetrate intact skin unaided. That is why skin infections are usually associated with breaches of skin caused by wounds, burns, or insect bites (Table 2-4). See Box 2-1 for a few examples of infections that changed the course of history due to the dire consequences of breaches in barrier defenses resulting from wounds.

Why is intact skin such an effective barrier to bacterial invasion? A number of characteristics combine to make skin inhospitable to bacterial growth, as well as difficult to penetrate (Figure 2-1). The epidermis consists of stratified

Table 2-4. Some consequences of breaching barrier defenses

TYPE OF BREACH	CONSEQUENCES
Venous catheters	Biofilm formation on the catheter; movement of bacteria into bloodstream
Burns	Infection of the burned tissue, sometimes followed by movement of bacteria into underlying tissue and blood
Damage to cornea caused by contact lenses	Infection of cornea
Respirator (tube inserted in the airway)	Allows bacteria to bypass defenses of upper airway
Perforation of the intestinal mucosa during surgery or other abdominal trauma	Release of bacteria into otherwise sterile tissue and blood
Buffering of stomach acid (achlorhydria)	Bacteria that cause intestinal infections are more likely to gain access to small intestine
Indwelling urinary catheter	Constantly drains bladder, eliminating washing action of urine; keeps sphincter open

squamous cells, most of which are **keratinocytes**. Keratinocytes produce the protein keratin, which is not readily degraded by most microorganisms. As cells from the dermis are pushed outward into the epidermal region, they produce copious amounts of keratin and then die. This layer of dead keratinized cells forms the surface of skin. The dead cells of the epidermis are continuously shed (**desquamation**). Thus, any bacteria that manage to bind to epidermal cells are constantly being removed from the body.

Skin is dry and slightly acidic (pH ~5), two features that inhibit the growth of many pathogenic bacteria, which prefer a wet, neutral (pH ~7) environment. Also, the temperature of skin (34 to 35°C) is lower than that of the body interior (37°C). Accordingly, bacteria that succeed in colonizing skin must be able to adapt to the very different internal environment of the body if they manage to reach underlying tissue. Interestingly, the causative agent of leprosy, *Mycobacterium leprae*, has an optimal growth temperature of 35°C, which may account for its predilection for the skin and mucosa of the upper respiratory tract.

Hair follicles, sebaceous (fat) glands, and sweat glands are composed of simple epithelial cells and offer sites for potential breaches in the skin that could be used by some bacteria to move past the skin surface. These sites are normally protected by peptidoglycan-degrading lysozyme (Figure 2-5) and by lipids that are toxic to many bacteria. However, some pathogenic bacteria are capable of infecting hair follicles or sweat glands, which is why skin infections such as boils (furuncles) and acne (pustules) are commonly centered at hair follicles.

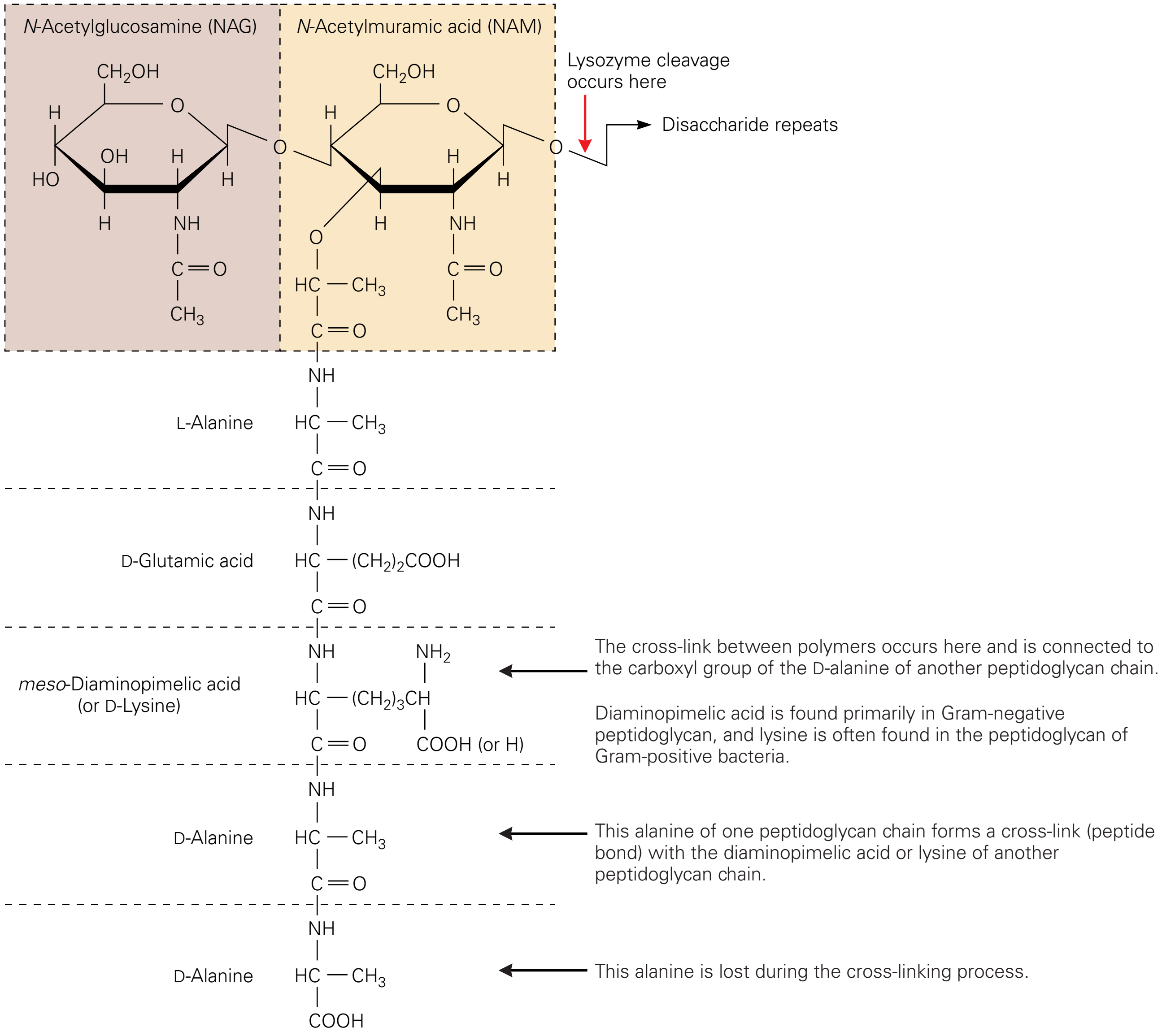
Defenses of Mucosal Surfaces

The respiratory, gastrointestinal, and urogenital tracts are topologically inside the body, but they are exposed constantly to the outer environment and foreign materials. Unlike the many dead layers found in skin, internal surface areas (called mucosal epithelia) are comprised of only one epithelial layer. The role of mucosal epithelial cells is absorption or secretion, so they are continuously bathed in fluids, having a temperature of around 37°C and a pH of 7.0 to 7.4. These warm, neutral, and moist conditions are ideal for growth of bacteria. To protect from bacterial colonization, these vulnerable epithelia have evolved a formidable array of chemical and physical barriers (see Table 2-2).

Mucosal cells are regularly replaced, with old cells sloughed off into the lumen. In fact, mucosal cells are one of the fastest dividing populations of cells in the body. Thus, bacteria that do manage to reach and colonize a mucosal surface are constantly eliminated from the mucosal surface and can only remain in the area if they can grow rapidly enough to colonize newly produced cells. Chemical and other innate defenses help reduce the growth rates of bacteria sufficiently to allow ejection of mucus blobs and sloughing of mucosal cells to clear the bacteria from the area.

An important protection of many internal epithelia is mucus. Mucus is a mixture of secreted glycoproteins (**mucin**) produced by **goblet cells**, a specialized glandular, modified columnar epithelial cell type incorporated into the epithelial layer. (The basic structure of glycoproteins is reviewed in Box 3-1). Mucus has a viscous, slimy consistency, which allows it to act as a lubricant. It also plays a protective role because it traps bacteria and prevents them from reaching the surface of the epithelial cells. Mucus is constantly being produced, and bacteria trapped in the mucus blobs are shed from the site. In the gastrointestinal and urinary tracts, peristalsis and the rapid flow of liquids through the area removes the mucus blobs along with the lumen contents. Indeed, infections can occur when this flow is disrupted, as may occur in the case of bedridden patients with urethral catheterization, which keeps the urethral entrance open and does not allow for flushing action, instead causing a slow, constant drain.

Another protective role of mucus is to bind proteins that have antibacterial activity (Table 2-2). Lysozyme is a major host defense protein found in tears, saliva, milk, and mucus and is also secreted by **Paneth cells** found at the bottom of crypts along the small intestine. Lysozyme is an enzyme


N-Acetylglucosamine (NAG)
N-Acetylmuramic acid (NAM)
CH2OH
O
H
OH
HO
NH
C=O
CH3
HC—CH3
Lysozyme cleavage occurs here
Disaccharide repeats
L-Alanine
D-Glutamic acid
HC—(CH2)2COOH
meso-Diaminopimelic acid (or D-Lysine)
NH2
HC—(CH2)3CH
COOH (or H)
D-Alanine
D-Alanine
COOH

The cross-link between polymers occurs here and is connected to the carboxyl group of the D-alanine of another peptidoglycan chain.

Diaminopimelic acid is found primarily in Gram-negative peptidoglycan, and lysine is often found in the peptidoglycan of Gram-positive bacteria.

This alanine of one peptidoglycan chain forms a cross-link (peptide bond) with the diaminopimelic acid or lysine of another peptidoglycan chain.

This alanine is lost during the cross-linking process.

Figure 2-5. Action of lysozyme. Lysozyme targets the peptidoglycan in bacterial cell walls of mainly Gram-positive bacteria and hydrolyzes the link between N-acetylmuramic acid and N-acetyl-D-glucosamine. It can also degrade the peptidoglycan of Gram-negative bacteria if the outer membrane is first disrupted by bile salts.

that targets the peptidoglycan of the bacterial cell wall and hydrolyzes the linkage between N-acetylmuramic acid and N-acetyl-D-glucosamine (Figure 2-5). It is most effective against the cell walls of Gram-positive bacteria, but it can also digest and weaken the cell walls of Gram-negative bacteria, especially if membrane-disrupting substances, such as the detergent-like bile salts found in the intestine, first breach the outer membrane. **Phospholipase** is an enzyme found in tears and secreted by Paneth cells that degrades the cytoplasmic membrane to lyse bacterial cells. **Lactoferrin** is an iron-binding, secreted protein found in mucus that sequesters iron and deprives bacteria of this essential nutrient.

Lactoperoxidase is another protein with antibacterial activity that is found in secretory fluids (e.g., milk, tears, saliva, and airway mucus). Lactoperoxidase is a heme-containing peroxidase that uses hydrogen peroxide (H_2O_2) as an oxidant to generate highly reactive oxygen species that kill bacteria. An important substrate is thiocyanate (SCN^-), which is converted into hypothiocyanite ($OSCN^-$), a potent bactericidal reagent. It is believed that exposed thiol groups (-SH) of enzymes and other proteins on the bacterial membrane surface are the primary targets of these reactive oxidants, which oxidize the thiols and thereby disrupt the normal function of the bacterial surface proteins.

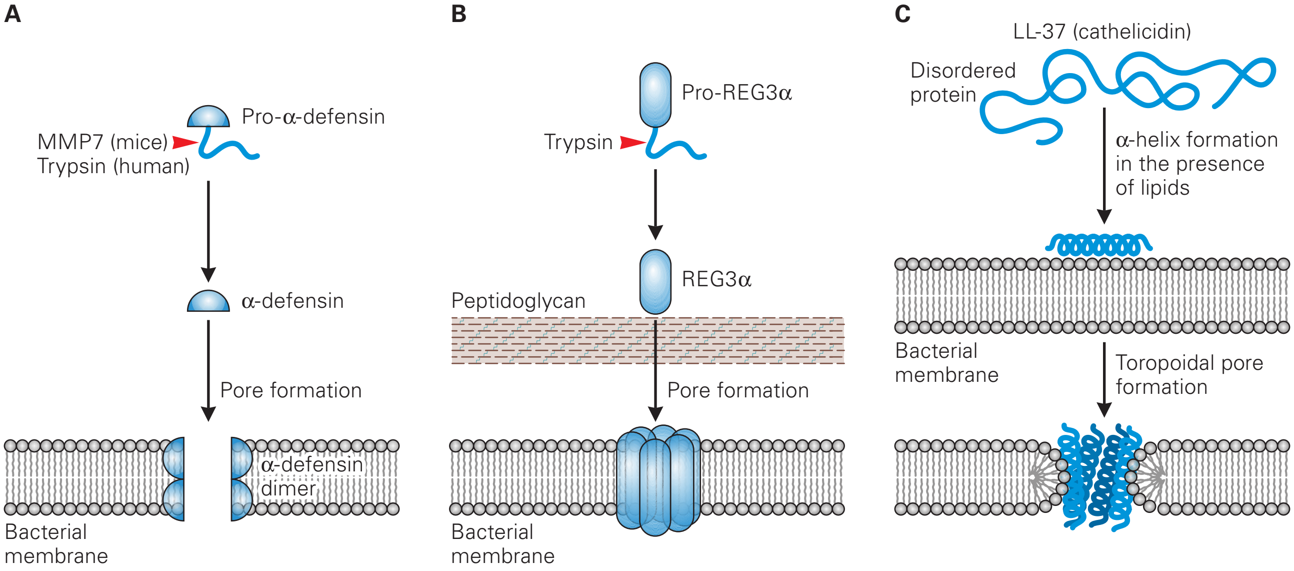


Figure 2-6. Three types of bacterial membrane permeabilization by gut antimicrobial peptides. Several antimicrobial peptides (AMPs) secreted by epithelial and Paneth cells in the intestine kill bacteria by forming pores in membranes. (A) α-defensin is expressed as an inactive propeptide that is processed and activated by proteases (MMP7 in mice and trypsin in humans). (B) REG3α is expressed as an inactive propeptide that is processed by proteolytic cleavage. The activated REG3α then binds to bacterial peptidoglycan and oligomerizes to form a hexameric pore in bacterial membranes. (C) Cathelicidins, such as LL-37, first bind to bacterial membranes via electrostatic interactions, then form α-helical structures in the presence of lipids and insert into the bacterial membrane to form pores. Adapted from Mukherjee S, Hooper LV. 2015. *Immunity* **42**:28–39, with permission.

Paneth cells also secrete toxic **antimicrobial peptides** (Figure 2-6), such as **defensins** (known as cryptdins in mice), **cathelicidins**, and **histatins**, which contain highly cationic (basic) regions that enable them to interact with negatively charged phospholipids and interact with or insert into bacterial cell membranes. These defensins kill bacteria by forming channels or holes in their membranes and depolarizing the cell by collapsing the proton-motive force (the potential energy stored as an electrochemical gradient across a membrane) that is essential for bacterial survival. This type of activity is responsible for the effectiveness of one of the first antibiotics, gramicidin, which is a pore-forming protein that kills bacteria. Defensins and other antimicrobial peptides have been found in the mouth, on the tongue, on skin, in the vagina, in the lungs, and in the crypts of the small and large intestines.

In the mouth, defensins may be the reason why infections of the tongue are so rare and why animals lick wounds. In the crypts of the intestinal mucosa, they are presumably protecting the intestinal stem cells, which divide constantly to replenish the cells of the intestinal mucosa. These peptides probably have some antibacterial effects that protect these locations from bacteria. It is worth keeping in mind, however, that the membrane-disrupting activity of these peptides can be inhibited by physiological salt concentrations or by serum. Consequently, their most important antibacterial activities may be exerted largely in another location where they are found: vacuoles inside phagocytic cells that engulf and kill bacteria.

Special Defenses of the Gastrointestinal Tract

Different regions of the gastrointestinal tract (Figure 2-7) have special antibacterial features that serve as barriers to pathogens. The lumen of the stomach has an extremely acidic environment (pH ~2). It was previously thought that most bacteria could not survive there, but scientists have found DNA from 128 different species of bacteria occupying the stomach. The only bacterium inhabiting this harsh environment that has been studied in detail is *Helicobacter pylori*, which causes gastritis, gastric ulcers, and even gastric cancer. In fact, *H. pylori* does not actually live in the lumen of the stomach, but rather in the mucin layer that covers and protects the stomach lining. Cells in the stomach lining secrete carbonate, which buffers the mucin layer to near neutral pH. *H. pylori* has the ability to protect itself long enough to reach the mucin layer and thus is not killed as readily as many bacteria are when they are exposed to the highly acidic environment of the stomach.

Other bacteria, including a number of foodborne pathogens (e.g., *E. coli, Salmonella,* and *Campylobacter*), have a response to acid that makes them better able to survive for short periods of time at pH 4 (**acid tolerance response**). This is higher than the pH of the stomach interior, so how could it be protective? One speculation is that bacteria ingested in foods are protected from the full impact of stomach acid by the buffering capacity of the food, although they would still be exposed to conditions well below pH 7. Even though they still cannot survive in the stomach for prolonged periods of time, the bacteria capable of mounting an acid

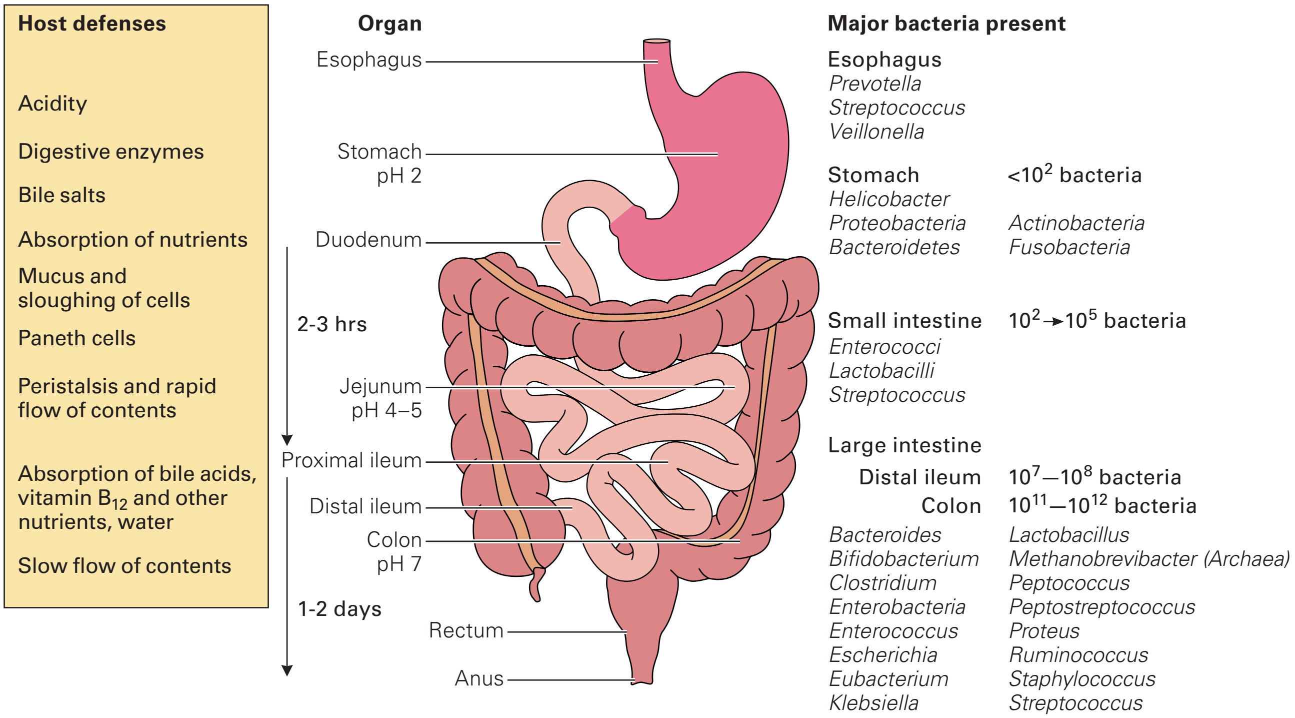

Host defenses
Acidity
Digestive enzymes
Bile salts
Absorption of nutrients
Mucus and sloughing of cells
Paneth cells
Peristalsis and rapid flow of contents
Absorption of bile acids, vitamin B_{12} and other nutrients, water
Slow flow of contents
Organ
Esophagus
Stomach pH 2
Duodenum
2-3 hrs
Jejunum pH 4–5
Proximal ileum
Distal ileum
Colon pH 7
1-2 days
Rectum
Anus
Major bacteria present
Esophagus
Prevotella
Streptococcus
Veillonella
Stomach
<10^2 bacteria
Helicobacter
Proteobacteria
Bacteroidetes
Actinobacteria
Fusobacteria
Small intestine
$10^2 \rightarrow 10^5$ bacteria
Enterococci
Lactobacilli
Streptococcus
Large intestine
Distal ileum
10^7—10^8 bacteria
Colon
10^{11}—10^{12} bacteria
Bacteroides
Bifidobacterium
Clostridium
Enterobacteria
Enterococcus
Escherichia
Eubacterium
Klebsiella
Lactobacillus
Methanobrevibacter (Archaea)
Peptococcus
Peptostreptococcus
Proteus
Ruminococcus
Staphylococcus
Streptococcus

Figure 2-7. The gastrointestinal tract. Different types and quantities of bacteria, mostly strict or facultative anaerobes, colonize along the gastrointestinal tract as the environments change with pH, bile, mucus content, digestive enzymes, and transit time. Data from Sartor RB. 2008. *Gastroenterology* **134**:577–594.

tolerance response may be able to survive long enough to reach the small intestine.

That many bacteria do not survive passage through the stomach to areas more favorable for bacterial growth, like the small intestine and colon, shows how the acidic environment of the stomach lumen does not just contribute to the digestion of food, but also acts as a protective barrier. Another indicator of the protective effect of the acidic environment of the stomach is that people who have achlorhydria, a condition that results in a less acidic stomach pH, have increased susceptibility to infections of the lower intestinal tract. Likewise, the U.S. **Food and Drug Administration (FDA)** has cited the widespread use of proton-pump inhibitor (PPI) drugs that block gastric acid production and reduce stomach acidity as a potential risk for increased intestinal infection.

For those bacteria that manage to survive the acid barrier of the stomach, **bile salts** await them in the small intestine and colon. Bile salts are steroids with detergent-like properties that are produced in the liver, stored in the gallbladder, and then released through the bile duct into the intestine when food is passing through. Bile salts help neutralize the stomach acid and are used to emulsify lipids in food to enable fat digestion and absorption through the intestinal wall. The detergent-like properties of bile salts help disrupt bacterial membranes, especially those of Gram-negative bacteria.

An equally important protection of the small intestine is the rapid flow of contents through the small intestine. This rapid flow, together with the bile salts and the rapid turnover of intestinal mucosal cells, helps keep high concentrations of bacteria from developing in the small intestine. High concentrations of bacteria would not only increase the chance that bacteria could invade the small intestinal mucosa, but also allow bacteria to compete more effectively for the nutrients (e.g., simple sugars, amino acids) that the small intestine is designed to absorb. In the colon, the flow rate of contents is drastically reduced compared to the flow rate in the small intestine. Some scientists have compared the difference to the passage from a rapidly flowing stream (small intestine) to a nearly stagnant pond (colon).

The importance of the rapid flow rate of contents through the small intestine as a protection against bacterial colonization is underscored by the fact that bacterial pathogens that cause intestinal infections such as gastroenteritis (diarrhea and pain) generally are able to swim to the mucosa of the small intestine and attach to the mucosal cells, thus keeping them from being washed out of the colon. Another illustration of the importance of rapid flow of contents is the fact that people who develop blind loops, or regions of outpouching that have rather stagnant contents, have problems due to the buildup of bacteria within those regions. At one time, the intentional surgical introduction of blind loops in the small intestine was tried as a means of weight reduction. Not surprisingly, a side effect of this in some people was the development of sepsis caused by an invasion of some of the bacteria that reached high concentrations in

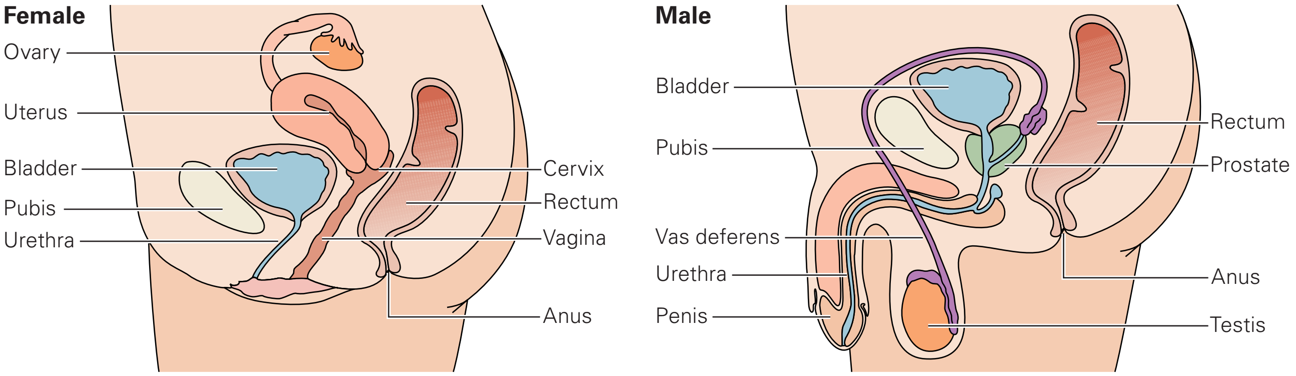


Figure 2-8. Comparison of the female and male urogenital tracts. Anatomical differences between the female and male urogenital tracts result in different environments and physical barriers that lead to different bacteria colonizing different sites. Physical barriers include a sphincter at the opening of the urethra that prevents ascent of microbes to the bladder and kidney. The urethra is shorter in females than males. Flushing of the bladder and urethra with urine removes adherent bacteria. Secretion of mucin blocks microbes from gaining access to the epithelial surface. A cervical mucus plug protects the uterus and fallopian tubes in females. *Lactobacillus* species, major resident bacteria in healthy females, protect the vaginal cavity from other bacteria that might enter from the anal area, which is relatively close to the vaginal opening. Sexually active partners exchange their resident microbiota with each other.

the blind loop and were thus better able to penetrate the fragile mucosal layer.

Special Defenses of the Urogenital Tract

The female and male urogenital tracts (Figure 2-8) offer different environments and so different bacteria are associated with the different areas. As with other mucosal areas, the epithelial layer of the urinary tract system (the kidneys, ureters, bladder, and urethra) is protected by secretion of mucin, blocking the bacteria from gaining access to the surface. The bladder is typically sterile in both males and females, and a number of defenses protect the urinary tract. The urethra sphincter prevents further ascent of the bacteria to the bladder and kidney. Urine itself is antiseptic, and flushing of the bladder with urine helps remove bacteria that manage to ascend the urethra. The prostate gland in men secretes defensins.

Altered conditions, such as pH changes or obstructions, can facilitate certain pathogens (e.g., *E. coli, Proteus mirabilis, Staphylococcus saprophyticus,* and *Klebsiella* species) to colonize the urethra and cause urinary tract infections (UTIs), especially in women. Almost 95% of UTIs are attributed to bacteria that normally reside at the opening of the urethra and travel up to the bladder and occasionally as far as the kidney. Women are more likely to get UTIs because in females the urethra is much shorter and closer to the anus. Some anatomical obstructions of the ureter or the urethra, such as prostate enlargement, kidney transplant, or bladder or kidney dysfunction, as well as indwelling catheter use, can impair bacterial clearance by urine and promote bacterial growth and infection.

In the vagina, a cervical plug protects the uterus and fallopian tubes from invasion by bacteria. The vagina is lined by a stratified epithelium, which produces a variety of protective defense mechanisms, including lysozyme, lactoferrin, small antimicrobial cationic peptides, and proteins (such as secreted IgA and IgG antibodies, secretory leukocyte protease inhibitors, and various cytokines and chemokines). The resident microbiota plays an important protective function against pathogens in the female genital tract. The application of molecular sequencing technologies over the past decade has contributed greatly to our understanding of urogenital microbiome composition and dynamics. In sexually active partners, the microbiota exchange freely and influence each other. There are also age-specific changes to the microbiomes and immune functions that occur, especially in response to hormones in women. Resident vaginal bacteria are thought to help prevent acquisition of sexually transmitted infections, such as HIV.

Lactobacillus species are the major resident vaginal bacteria found in healthy humans. *Lactobacillus* bacteria ferment glycogen, which is abundantly produced in the vaginal tract. These bacteria produce hydrogen peroxide and lactic acid, which helps maintain a weakly acidic (pH 4–5) environment that serves to inhibit the growth of other microbes, thereby preventing infection by yeast (vulvovaginal candidiasis or vaginitis) and bacteria (bacterial vaginosis). Humans are unique among primates in having a vaginal microbiome dominated by *Lactobacillus* species, though it is not known why. How the vaginal mucosa tolerates chronic colonization with *Lactobacillus* species, while still protecting against colonization by other microbes, is an area of active research.

Special Defenses of the Respiratory Tract

The respiratory system is comprised of a ciliated epithelial cell layer that is interspersed with goblet cells that secrete mucin. In the respiratory tract (as well as in the fallopian tubes of the vaginal tract), there are specialized **ciliated columnar cells**, whose elongated protrusions (**cilia**) continuously wave in the same direction. The waving action of the

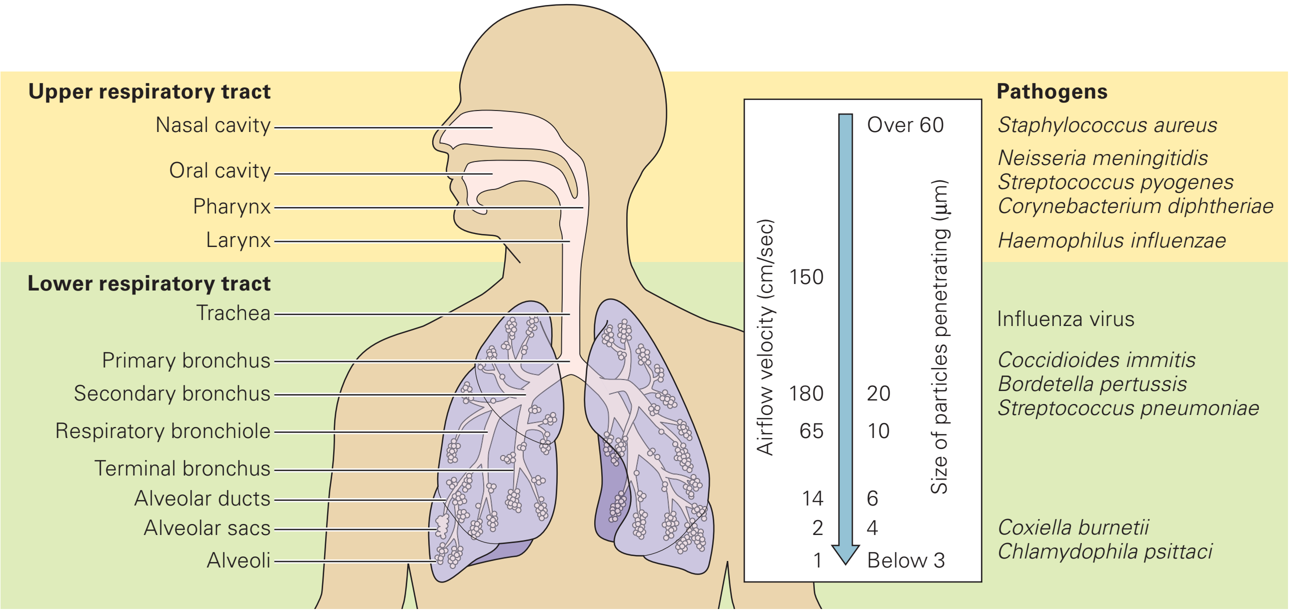

Upper respiratory tract
Nasal cavity
Oral cavity
Pharynx
Larynx
Lower respiratory tract
Trachea
Primary bronchus
Secondary bronchus
Respiratory bronchiole
Terminal bronchus
Alveolar ducts
Alveolar sacs
Alveoli
Airflow velocity (cm/sec)
150
180
65
14
2
1
Size of particles penetrating (µm)
Over 60
20
10
6
4
Below 3
Pathogens
Staphylococcus aureus
Neisseria meningitidis
Streptococcus pyogenes
Corynebacterium diphtheriae
Haemophilus influenzae
Influenza virus
Coccidioides immitis
Bordetella pertussis
Streptococcus pneumoniae
Coxiella burnetii
Chlamydophila psittaci

Figure 2-9. **The upper and lower respiratory tract.** The respiratory system is comprised of a ciliated epithelial cell layer that secretes mucus. Turbulence of airflow and mucociliary action help keep the lungs clear of particles and microbes. The upper respiratory tract includes the nose, nasal cavity, mouth, throat (pharynx), and voice box (larynx). The upper respiratory tract is often colonized by *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae*, *Moraxella catarrhalis,* and nontoxigenic *Corynebacterium diphtheriae*. These bacteria can be opportunistic pathogens under certain conditions or when they colonize the lower respiratory tract. The lower respiratory tract includes the trachea, bronchi, and lungs (alveoli). The lungs are normally clear of bacteria, but can be invaded by opportunistic or outright pathogens during infection or under certain conditions. Data from Madigan MT, Martinko JM, Stahl D, Clark DP. 2015. *Brock Biology of Microorganisms*, 13th ed. Pearson, Upper Saddle River, NJ.

cilia propels mucus blobs out of the area. Airway epithelial cells also secrete **collectins (collagen-containing C-type lectins)**, including surfactants A and D that bind to bacterial lipopolysaccharide (LPS), defensins and other antimicrobial peptides, and proteases. Hospitals often encounter problems associated with comatose patients, in which the absence of a normal cough reflex and reduced mucociliary clearance result in an increased susceptibility to respiratory infections. Turbulence of airflow from breathing, coughing, and sneezing serves to expel microbes out of the lungs, sometimes at velocities reaching over 150 cm/sec. In hospitals, problems can occur with respirators, which introduce air with tiny water droplets potentially contaminated with pathogens directly into the lung, thereby bypassing the upper respiratory airflow and mucociliary defenses.

The upper and lower respiratory tracts have different environments, and different commensal and pathogenic microbes are associated with the different areas (Figure 2-9). A healthy lower respiratory tract lacks significant microbiota, while a few bacteria normally colonize the upper respiratory tract, most notably *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae*, *Moraxella catarrhalis,* and nontoxigenic *Corynebacterium diphtheriae*, all of which are potential pathogens and are known to cause upper and lower respiratory infections when the immune system is impaired. In lung diseases such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease, other pathogens also colonize the lungs, including *Pseudomonas aeruginosa, Burkholderia cepacia,* and *Mycoplasma pneumoniae*. In humans, colonization of the lower respiratory tract with *Bordetella pertussis* always leads to disease symptoms of whooping cough.

Immune Defenses of the Skin and Mucosa

Although this chapter focuses on the protective physical and biochemical barriers provided by intact skin and mucosa, it is important to note that these barriers are backed up by specialized portions of the immune system, which will be described in more detail in subsequent chapters. For example, bacteria that manage to get past the epidermis through cuts or burns encounter a specialized cell type called **Langerhans cells**. Langerhans cells belong to a class of cells called **dendritic cells (DCs)** that process the invading bacteria and activate the immune cells of the **skin-associated lymphoid tissue** (**SALT**). We will discuss the role of phagocytes (e.g., macrophages and dendritic cells) in chapter 3 when we cover "The Innate Immune System: Always on Guard."

As was the case with skin, mucosal surfaces generally have an underlying population of phagocytic cells and immune cells. This mucosal defense system, which is distinct from the system that controls immune cells in blood, lymph nodes, and other organs, is called the **mucosa-associated lymphoid tissue** (**MALT**). The mucosal surface of the small intestine is underlain with the **gastrointestinal-associated lymphoid tissue** (**GALT**), while the lungs have **bronchial-associated lymphoid tissue (BALT)** and the upper respiratory tract has **nasopharyngeal-associated lymphoid tissue (NALT)**.

These mucosal defense systems at the interface of the innate and adaptive immune systems are composed of macrophages, T cells, B cells, and **M cells** (**microfold cells** that engulf gut lumen contents and present them to underlying antigen-presenting cells). Their primary function is to make **secretory IgA (sIgA)**, an antibody that is secreted into mucus. Antibodies are proteins that bind to specific sites on bacteria or other pathogens. sIgA is thought to increase the stickiness of mucin by attaching to mucin sugars at one end, leaving its two other antigen-binding ends free to bind and trap bacteria trying to reach the mucosal layer. The sIgA-trapped bacteria are then sloughed off along with the mucin. We will return to the role of MALT in chapter 4.

Models for Studying Breaches of Barrier Defenses

Animal models have been widely used to study skin, eye, and mucosal infections. Because some of these animal models involve breaches such as cuts or burns that can be damaging and painful, experimental protocols must include explicit plans to monitor and minimize pain and discomfort to the animals as much as possible and to minimize the number of animals needed to obtain statistically significant results. When available, validated alternative infection models that do not involve animals should be used. In addition, a committee with expertise in animal welfare and experimentation must first approve the rationale for experiments on animals and the detailed protocols themselves before the experiments are performed. Some of the ethical and procedural issues that lead to appropriate animal experimentation are discussed later in chapter 8. For continuity, some of the models used to study eye, skin, and mucosal infections are mentioned here without this context.

One of the earliest models for studying skin infections is the burned-rodent model. A patch of skin on an animal that is anesthetized is shaved and then burned with an alcohol flame. Just as is the case with human burns, bacteria that could not infect intact skin can infect the burned rodent tissue. The eye is another surface of the body that is remarkably resistant to infection. Eye infections of the sort seen in patients who have been careless with contact lenses or have suffered small cuts in the cornea are mimicked by a rabbit model, in which small shallow cuts are made in the cornea of an anesthetized animal's eye. Both of these models have been used extensively to study infections caused by *P. aeruginosa,* one of the main causes of burn and eye infections in humans.

In the previous chapter, some unusual lower animal models were mentioned. *Caenorhabditis elegans* (worms) and *Drosophila* (flies) are not very useful for studies of skin infections, because the "skin" of these organisms is chitinous rather than epithelial. The zebra fish is a better model, especially for studies of the mucosal defenses. More recently, infection models have been developed based on tissues from, for example, chicken embryos. Rodents have been widely used to investigate pathogens, such as *Salmonella,* that bind to the intestinal mucosa. In rodents, these pathogens can sometimes cause more invasive infections than they cause in humans, but the interaction between the bacteria and the mucosa can nonetheless be followed even in these cases. A rodent model has been developed in which autoclaved feces, inoculated only with the bacterium of interest, are implanted in the intra-abdominal area of the rodent to mimic the effects of surgical penetration of the colonic mucosa.

The impact of toxins, such as diarrhea-causing toxins, on the small intestine can be monitored by the **rabbit ileal loop model** (Figure 2-10). In this model, the small intestine of an anesthetized rabbit is tied off into 5- to 10-cm sections by suture, the toxin or toxin-producing bacterium is injected into one of the sections (loops), and the organ is placed back into the peritoneal cavity. Many diarrheal toxins cause water to be lost by the intestinal tissues into the lumen of the gut, and this can be observed by a swelling of the section into which the toxin was injected. After 12 to 24 hours, the animal is sacrificed and the loop length and fluid volume (in

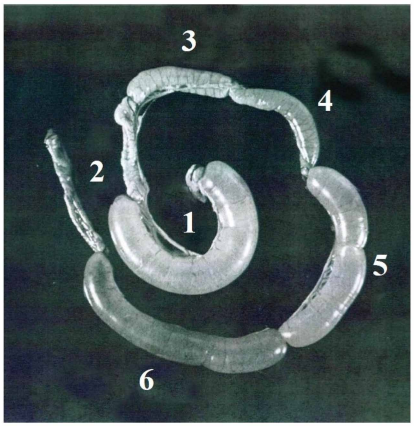


Figure 2-10. The rabbit ileal loop model of diarrheal disease. Shown are tied-off segments (loops) of rabbit ileal injected with culture filtrates from an *E. coli* strain producing cholera-like toxin that induces diarrhea. Loop 1 was injected with positive control solution of cholera toxin, loop 2 with negative control solution of phosphate-buffered saline, and loops 3 through 6 with increasing amounts of *E. coli* culture filtrates. After overnight exposure in the animal, the animal is sacrificed and the ileal loops are removed and examined for distension (swelling). Reproduced from Sack RB. 2011. *Indian J Med Res* **133:**171–180, with permission.

ml/cm) are measured as readout. Distension (i.e., swelling) of the ileal loop section indicates release of the fluid into the lumen of the segment as a result of toxin action.

Genetically engineered mice called transgenic mice or knockout mice are being increasingly used in experiments to probe the interaction of the normal microbiota and the intestinal mucosal cells because they have genes that have been altered or disrupted. Unexpectedly, some of the mice designed originally for studies of the immune system that were missing genes encoding the cytokines, interleukins IL-1 and IL-10 (see chapter 3), proved to be good models for a type of intestinal inflammation called **inflammatory bowel disease (IBD)**. The presence of the normal bacterial microbiota of the colon seems to be responsible for the inflammatory bowel condition seen in some of these mice.

These examples are given to provide an introduction to the types of animal models that are available for studying the protective features of skin and mucosa and the consequences of breaching these barriers. Additional animal models used in connection with studying bacterial diseases, as well as alternatives to animal models such as mammalian host cells cultured *in vitro* in the laboratory, will be described in chapter 8 and other chapters covering specific types of infectious diseases.

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Questions

1. In what sense are *S. epidermidis* infections an example of how changing human practices can provide new opportunities for bacterial pathogens? *S. epidermidis* is classified as an opportunist. Why is this the case?

2. Explain why infections of the skin occur more often in folds of the skin or under bandages than in regions of skin exposed to air.

3. How and why do the defenses of mucosal surfaces differ from those of the skin? How do they resemble each other?

4. Consider a bacterium that is ingested via contaminated water and locally colonizes the small intestine. What host defenses would hamper this type of colonization from occurring initially and from leading to an infection in an unimmunized person?

5. Resident microbiota are essential in preventing the colonization of pathogenic bacteria in certain parts of the body. Name the regions of the body where normal microbiota might be protective. Name some mechanisms by which they accomplish this protection. Name some organs that normally remain sterile to bacterial contamination and that do not contain a resident microbiota.

6. Why are people with indwelling catheters more susceptible to infection?

7. Explain the role that mucin plays in host defense.

8. Lysozyme is more effective against growing bacteria. Why might that be so? Why is lysozyme digestion often more effective against Gram-positive bacteria than Gram-negative bacteria?

3

IN THIS CHAPTER

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- Monocytes, Macrophages, and Dendritic Cells (DCs)
- Granulocytes: Basophils, Mast Cells, and Eosinophils
- Transmigration—How Do Phagocytes Know When and Where to Go?
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The Lymphatic System

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Questions

CHAPTER 3

The Innate Immune System

ALWAYS ON GUARD

Bacterial invaders can enter the blood and tissue at any time due to breaches in skin or mucosal surfaces. When this happens, the body needs a way to react quickly and in a relatively nonspecific way to those invaders, regardless of whether it has encountered them before. At the same time, the body must also have a way to distinguish the invading cells from its own cells, and cells that have been infected from those that have not. How would you design a "barcode" for bacteria? Even scientists with advanced degrees might be stumped by that question, but that is precisely what the human body has done using the cells of its immune system. The innate immune system jumps into action when the skin or mucosal barriers are breached, but it does so in a carefully controlled way—which is a good thing, because overreaction by this same defense system can cause considerable damage to the host. Finally, to ensure that all invaders are cleared from the body and that an even faster and more specific response is mounted at the next attempted invasion by that pathogen, the innate immune system prompts and primes the adaptive immune system.

Triggering Innate Immune Defenses

Skin and mucosal surfaces are highly effective in preventing pathogenic bacteria from entering the tissue and blood, but from time to time bacteria succeed in breaching these surfaces. We ended the previous chapter with a brief mention of the skin- and mucosal-associated lymphoid tissue (SALT and MALT, respectively) defense systems located at the interface between the external surface defenses and the underlying tissues and blood. When the skin and epithelial layers are breached, both SALT and MALT have critical functions in stimulating additional defenses, initially triggering the general **innate immune system** and then priming the more specific **adaptive immune system**. MALT activation also keeps microbes away from the vulnerable epithelial layer by producing **secretory immunoglobulin A (sIgA)** that binds to both mucin and the microbe and is then sloughed off and removed from the body. We will return to this aspect of MALT in chapter 4.

The most important function of the innate immune system is to quickly recognize and eliminate pathogens. The innate immune system is comprised of a subset of **white blood cells (leukocytes)** that include **phagocytic cells (phagocytes)**, granulocytic cells **(granulocytes)**, and **natural killer (NK) cells**. Because these cells are always present and ready to respond, they are the body's first responders to pathogen invasion. Together they help clear foreign elements from the body and process them for presentation to the adaptive immune system. The innate immune system becomes activated when it recognizes components of foreign invaders. For bacteria, this recognition is through shared repeated structural components and other molecules specifically found in bacteria. These quasi "bacterial barcodes" are termed **pathogen-associated molecular patterns (PAMPs)** and play an important role in initiating the innate immune responses by triggering the production and release of cytokines and chemokines from infected cells and from nearby immune cells. Additional proteins and peptides found in the circulatory system, collectively referred to as the **complement system,** help organize and regulate the activities of the phagocytes, NK cells, and other immune cells. Along with phagocytes and NK cells, the components of the PAMP recognition system and complement system are key players in detecting invaders and triggering the innate and adaptive immune responses (major players are listed in Table 3-1).

Innate Immune Cells That Defend Blood and Tissue

The major cell types comprising the innate immune system can be grouped into three categories based on their primary modes of action: phagocytes, granulocytes, and NK cells. Phagocytes are cells that ingest and kill foreign invaders. Granulocytes (**basophils**, **mast cells,** and **eosinophils**) promote the killing of invading pathogens by releasing proinflammatory cytokines and stimulating immune cells. NK cells are cytotoxic cells that act extracellularly to attack and kill host cells that are infected with bacteria or altered due to interaction with bacteria or their products. The characteristics of different phagocytes, granulocytes, and NK cells, as well as their relationships to each other and to cells of the adaptive immune defense system, are illustrated in Figure 3-1. We will begin our discussion with the phagocytes, which include **polymorphonuclear leukocytes** (also called **PMN**s or **neutrophils**—we will use these two terms interchangeably), **monocytes**, **macrophages,** and **dendritic cells (DCs)**.

Neutrophils (PMNs)

Neutrophils (PMNs) are the most abundant phagocytic cell in the body. Found circulating in the bloodstream, they are the first phagocytes that arrive at a site of infection, usually within 30 minutes, and immediately engulf (**phagocytose**) invading microbes and release antibacterial components stored in their granules that destroy the bacteria, including: cationic peptides that poke holes in membranes; proteases, lipases, and hydrolases that degrade bacterial surface proteins, membranes, and toxins, respectively; lysozyme that hydrolyzes the peptidoglycan of bacterial cell walls; myeloperoxidase that generates toxic reactive oxygen species; and lactoferrin that sequesters iron away from the bacteria. Neutrophils secrete signaling molecules: chemokines that attract nearby monocytes and cytokines that stimulate their conversion into macrophages. They can also produce and, when stimulated, rapidly (within minutes) release **neutrophil extracellular traps (NETs)**, networks of extracellular chromatin DNA fibers (chromosomal DNA with DNA-binding histones) that contain the proteases, elastase and cathepsin G, and myeloperoxidase. These NETs serve to trap and kill the bacteria extracellularly and prevent bacterial spread from the site of infection.

Monocytes, Macrophages, and Dendritic Cells (DCs)

Neutrophils, dendritic cells (DCs), and monocytes are derived from the same myeloid progenitor cells. In response to chemokines (chemical attractants) and other signals, monocytes and DCs leave the bloodstream and enter the site of infection. There, **cytokines** (stimulatory signaling molecules) released by damaged or infected cells trigger the monocytes to convert into macrophages, that are then activated by neutrophils at the site (see Table 3-2 and later in this chapter).

Macrophages and DCs are included here as members of the innate immune system, but they will appear again in chapter 4 as important members of the adaptive immune system. This is due to the fact that, upon first exposure to a particular pathogen, macrophages and DCs act to ingest and kill bacteria in a nonspecific way through the innate immune response, while also presenting pieces of the pathogens (**antigens**) to cells of the adaptive immune system. Upon subsequent exposure to a pathogen, monocytes and macrophages can also phagocytose invading bacteria that are coated with antibodies generated through the adaptive immune response. Macrophages engulf and digest cellular debris, microbes, foreign particles, infected or diseased host cells, and any other cell that does not possess the types of recognition surface molecules that indicate it is a healthy host cell.

Monocytes circulating in the blood have a half-life of about one day, but once they migrate from the circulating blood into tissues, they differentiate into different types of macrophages that become residents at the tissue sites, where they can survive for several months or years. Some examples of resident macrophages are the **Kupffer cells** of the liver, **alveolar macrophages** of the lung, and **spleen macrophages**. Macrophages can either stimulate the immune system and cause inflammation, or dampen the immune system and decrease inflammation through the release of specific signaling molecules (cytokines), depending on whether they are M1 macrophages or M2 macrophages, respectively. M1

Table 3-1. **Components of the innate immune system and their activities**

COMPONENT	CHARACTERISTICS	FUNCTION(S)
Phagocytic cells		
PMNs (neutrophils)	Found in the bloodstream; most common phagocyte in body; short-lived; have granules that contain antimicrobial peptides, proteases, lysozyme, myeloperoxidase, and lactoferrin	Phagocytose microbes and release contents of granules that destroy them; generate neutrophil extracellular traps (NETs) that ensnare microbes extracellularly; secrete cytokines that stimulate monocytes and macrophages
Monocytes	Produced in bone marrow; found in blood; migrate to tissue where infection is occurring	Precursor of macrophages that mature into macrophages and DCs when they migrate into tissues in response to chemokines (GM-CSF for macrophages; IL-4 and GM-CSF for DCs) and are activated by cytokines and interaction with microbes
Macrophages		Take up and process bacteria for antigen presentation (similar to DCs); produce cytokines; activate T cells and B cells
Kupffer cells	Resident macrophages in liver	
Alveolar macrophages	Resident macrophages in lungs	
Spleen macrophages	Resident macrophages in spleen	
Natural killer cells (NKs)	Found in blood and tissue	Recognize and kill infected or altered host cells; produce cytokines; organize response to infection
Dendritic cells (DCs)	Found as resident DCs in tissues that are in contact with the external environment (skin, nose, lungs, stomach, intestines) and as more immature DCs circulating in blood and lymph; similar in morphology and function to monocytes and macrophages	Take up and process bacteria (similar to macrophages); present bacterial antigens to immune cells to initiate adaptive immune response; activate T cells and B cells
Langerhans cells	Resident DCs in skin and mucosa; derive from monocytes in response to colony stimulating factor-1 (CSF-1)	
Lymph node DCs	Resident DCs in lymph	
Proteins/Peptides		
Complement	Produced in liver; found in blood and tissue	Components enhance opsonization of bacteria by phagocytes; attract PMNs and monocytes to site of infection; stimulate inflammatory responses; stimulate vasodilation; directly disrupt and kill Gram-negative bacteria
Cytokines	Produced by phagocytes (PMNs, macrophages, DCs, NK cells), other immune cells (T cells, B cells, mast cells, basophils), and other host cells (epithelial cells, endothelial cells, fibroblasts) that have been exposed to or invaded by bacteria; released by stimulation of complement system	Small (5–20 kDa) peptides and proteins that are secreted and bind to receptors to regulate responses of phagocytes (PMNs, monocytes), T cells and B cells, and other immune cells
Chemokines	Produced by phagocytes, DCs, and other host cells that have been exposed to or invaded by bacteria; released by stimulation of complement system	Small (8–10 kDa) peptides that are secreted and bind to receptors to stimulate chemotaxis of phagocytes (PMNs, monocytes) and transmigration to site of infection
Transferrin	Produced in liver; found in blood and tissues	Glycoprotein that binds tightly to iron (high affinity for Fe^{3+} ions) and sequesters iron in blood to regulate free iron concentrations in body fluids
Lactoferrin	Produced in liver; secreted in milk, saliva, tears, and nasal fluids, also found in blood plasma and neutrophil granules	Glycoprotein that binds tightly to iron (high affinity for Fe^{3+} ions) and sequesters iron in extracellular fluids to regulate free iron concentrations in body fluids through cellular uptake via cell surface transferrin receptors

(*continued*)

Table 3-1. **Components of the innate immune system and their activities (*continued*)**

COMPONENT	CHARACTERISTICS	FUNCTION(S)
Calprotectin	Produced and secreted by neutrophils (constitutes up to 60% of soluble protein in cytosol of neutrophils); also found in monocytes, macrophages, and epithelial cells	Calcium-binding protein dimer that in the presence of calcium binds very tightly to manganese and zinc
Cationic antimicrobial peptides (defensins, cathelicidins)	Found in granules of neutrophils and NK cells; lysosomes of monocytes, macrophages, mucosal epithelial cells; secreted by Paneth cells of small intestine	Small (12–80 amino acids), cysteine-rich cationic peptides that bind and insert into microbial cell membranes, forming pores and disrupting cell membranes
Lysozyme (muramidase, N-acetylmuramide glycanhydrolase)	Found abundantly in secretions such as tears, saliva, milk, and mucus; also present in cytoplasmic granules of macrophages and PMNs	Glycoside hydrolase enzyme of 129 amino acids (14.3 kDa) that damages growing bacterial cell walls by hydrolyzing the 1,4-β-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues of peptidoglycan

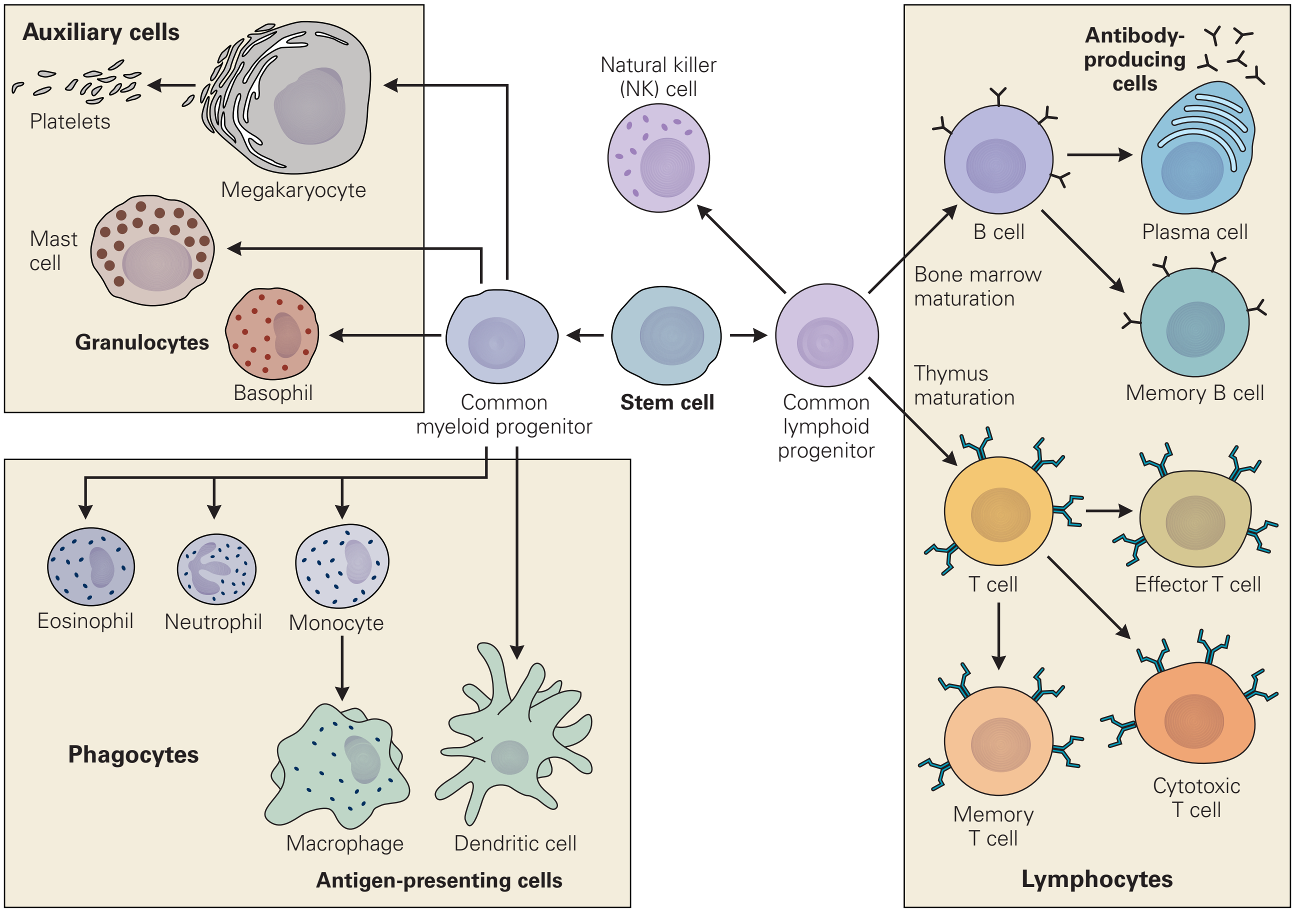

Auxiliary cells
Platelets
Megakaryocyte
Mast cell
Granulocytes
Basophil
Natural killer (NK) cell
Common myeloid progenitor
Stem cell
Common lymphoid progenitor
Antibody-producing cells
B cell
Plasma cell
Bone marrow maturation
Memory B cell
Thymus maturation
T cell
Effector T cell
Memory T cell
Cytotoxic T cell
Lymphocytes
Eosinophil
Neutrophil
Monocyte
Phagocytes
Macrophage
Dendritic cell
Antigen-presenting cells

Figure 3-1. **Characteristics and differentiation of the various types of leukocytes of the human body.** Leukocytes can be divided into three groups: auxiliary cells (platelets, megakaryocytes, mast cells, and basophils); phagocytes (neutrophils, eosinophils, monocytes, macrophages, and dendritic cells); and two types of lymphocytes: B cells (antibody-producing B cells and plasma cells, memory B cells), and T cells (cytotoxic T cells [CTLs], effector T cells [helper Th1/Th2/Th17 cells, regulatory Treg cells], and memory T cells).

Table 3-2. **Selected immune cytokines/chemokines and their activities**

CYTOKINE	PRODUCING CELL(S)	TARGET CELL(S)	FUNCTION
GM-CSF	Macrophages, Th cells, mast cells, NK cells, endothelial cells, fibroblasts	Progenitor (stem) cells	Immune cell growth factor that stimulates stem cells to differentiate into neutrophils, eosinophils, basophils, and monocytes; induces monocytes to transmigrate from circulation to tissues and mature into macrophages and DCs
IL-1α	Activated macrophages, PMNs, constitutively expressed in epithelial and endothelial cells as precursor	Th cells, CD4+ cells, mature B cells; fibroblasts, smooth muscle cells, keratinocytes; co-stimulates CD8$^+$ cells	IL-1α precursor is cleaved by calpain (calcium-activated cysteine protease associated with plasma membrane) into mature 18-kDa cytokine that activates lymphocytes; binds to IL-1 receptor; stimulates production of IL-2 and TNF-α; stimulates proliferation of fibroblasts, keratinocytes, and CD4+ cells; IL-1a and TNF induce fever and inflammation
IL-1β	Expressed in activated macrophages, DCs, B cells as precursor	Activates lymphocytes	IL-1β precursor is cleaved (processed) into mature 17.5 kDa peptide by cytosolic caspase-1 protease; mediates inflammatory responses from activation of inflammasome
IL-2	Th1 cells	Activates T and B lymphocytes, NK cells	Cytokine that binds IL-2 receptor on lymphocytes, particularly Th cells; promotes differentiation and proliferation of T cells into regulatory T cells, effector T cells, and memory T cells
IL-3	Activated Th cells; NK cells, basophils	Hematopoietic stem cells, mast cells	Cytokine that binds IL-3 receptor and stimulates differentiation of hematopoietic stem cells into myeloid progenitor cells or in combination with IL-7 into lymphoid progenitor cells; stimulates, along with GM-CSF, erythropoietin (EPO) and IL-6 proliferation of myeloid cells (granulocytes, monocytes, DCs)
IL-4	Th2 cells; basophils	Th0 cells; Th2 cells; B cells, macrophages	Cytokine that binds IL-4 receptor and induces differentiation of naïve Th cells (Th0 cells) into Th2 cells; stimulates Th2 cells to produce more IL-4; activation of macrophages into M2 macrophages; B cell class switching into IgE-producing B cells; up-regulates MHC II production; stimulates proliferation of B cells and T cells
IL-5	T cells, mast cells	B cells; eosinophils	Cytokine triggers activate B cells and eosinophils to terminally differentiate; stimulates B cell growth; increases antibody secretion; is associated with severe allergic reactions due to activation of eosinophils
IL-6	T cells; macrophages; osteoblasts	Smooth muscle cells; osteoclasts	Proinflammatory cytokine that binds IL-6 receptor and mediates fever (crosses blood–brain barrier to increase prostaglandin E2 synthesis in hypothalamus, which increases body temperature), secreted by macrophages in response to PAMPs; stimulates differentiation of osteoclasts; also acts as myokine produced by muscle cells during exercise, where it increases production of anti-inflammatory IL-1 receptor antagonist (IL-1Ra) and IL-10
IL-7	Stromal cells in bone marrow and thymus; keratinocytes, DCs, hepatocytes, neurons; epithelial cells	Hematopoietic stem cells; T cells, B cells, NK cells	Cytokine that binds to IL-7 receptor and stimulates along with IL-3 differentiation of hematopoietic stem cells into lymphoid progenitor cells, stimulates proliferation of B cells, T cells, and NK cells
IL-8	Macrophages, epithelial and endothelial cells, other cells with TLR receptors	PMNs, macrophages, mast cells, endothelial cells, keratinocytes	Chemokine that recruits PMNs and other granulocytes; induces phagocytosis; promotes angiogenesis
IL-9	CD4$^+$ Th cells	Hematopoietic cells	Binds IL-9 receptor and stimulates cell proliferation (prevents apoptosis)
IL-10	Monocytes, Th2 cells, regulatory T cells (Treg), mastocytes, cytotoxic T cells	Macrophages; regulatory T cells; B cells	Anti-inflammatory cytokine that binds to IL-10 receptor and inhibits expression of IL-1, IL-3, GM-CSF, IFN-γ, and TNF-α; suppresses antigen presentation by macrophages and DCs; stimulates B cells to produce antibodies
IL-12	Macrophages, DCs, PMNs	Activated cytotoxic T cells, NK cells	T cell-stimulating factor that binds IL-12 receptor and induces differentiation of naïve T cells into Th1 cells; stimulates production of IFN-γ and TNF-α from T cells and NK cells; enhances cytotoxic activity of CD8$^+$ cytotoxic T cells and NK cells

(*continued*)

Table 3-2. Selected immune cytokines/chemokines and their activities (*continued*)

CYTOKINE	PRODUCING CELL(S)	TARGET CELL(S)	FUNCTION
IL-13	Th2 cells	B cells, intestinal epithelial cells	Proinflammatory cytokine that mediates allergic inflammatory responses such as stimulation of IgE secretion from B cells
IL-15	PMNs; monocytes, macrophages, DCs, keratinocytes, fibroblasts, nerve cells	NK cells, T cells	Cytokine that binds IL-15 receptor and induces cell proliferation of NK cells; produced in response to PAMPs; activates and stimulates proliferation of T cells and NK cells
IL-17	CD4$^+$ Th cells (especially Th17 cells)	Macrophages, keratinocytes, fibroblasts, endothelial and epithelial cells	Proinflammatory cytokine that binds to IL-17 receptor and mediates delayed-type reactions by increasing production of many other cytokines (IL-6, GM-CSF, IL-1β, TGF-β, TNF-α, and IL-8) in various tissues that recruit monocytes and PMNs to site of inflammation; induces antimicrobial peptide production by epithelial cells; commonly associated with allergic responses
IL-21	Activated CD4$^+$ Th cells (particularly in Th2 and Th17 cells), NK cells	NK cells, cytotoxic CD8$^+$ T cells	Cytokine binds IL-21 receptor and induces proliferation of cytotoxic T cells and NK cells
IL-33	Th2 cells, mast cells, basophils, eosinophils, DCs, macrophages, osteoblasts, endothelial and epithelial cells, fibroblasts	Th2 cells, mast cells	Induces cytokine (IL-4, IL-5, IL-9, IL-13) production from Th2 cells
IFN-α	Leukocytes (DCs)	Various immune cells and infected cells	Antiviral activity, produced in response to PAMPs, stimulates production of chemokines
IFN-β	Fibroblasts	Various immune cells and infected cells	Antiviral activity, produced in response to PAMPs, stimulates production of chemokines
IFN-γ	NK cells, CD4$^+$ Th1 cells, CD8$^+$ cytotoxic T cells, activated DCs, activated macrophages	Macrophages; Th2 cells, NK cells	Released by Th1 cells when activated by IL-12; block proliferation of Th2 cells; potent activator of macrophages; increases expression of MHC I in all cells and MHC II in immune cells; promotes differentiation of naïve CD4$^+$ Th0 cells into Th1 cells; stimulates leukocyte transmigration
MIP-1α (CCL3)	Macrophages, DCs, T cells and B cells	Granulocytes (neutrophils, eosinophils, basophils), monocytes, macrophages, DCs	Macrophage inflammatory protein that acts as a chemokine for granulocytes, monocytes, macrophages, DCs, CD8$^+$ T cells; produced in response to LPS, LTA; binds to CCR1, CCR4, and CCR5 receptors and induces expression of proinflammatory cytokines (IL-1, IL-6, and TNF-α) from macrophages and fibroblasts
MIP-1β (CCL4)	Macrophages, DCs, T cells and B cells	NK cells, monocytes, DCs	Macrophage inflammatory protein that acts as a chemokine for NK cells, monocytes, DCs, CD4$^+$ T cells; produced in response to LPS, LTA; binds to CCR5 receptor and induces expression of proinflammatory cytokines (IL-1, IL-6, and TNF-α) from macrophages and fibroblasts
TGF-β	All leukocytes	T cells, B cells, macrophages, monocytes	Transforming growth factor is a cytokine that controls proliferation and differentiation of T cells, B cells, macrophages, and monocytes; released by regulatory Treg cells to inhibit other T cells, inhibits proliferation of activated T cells, inhibits secretion of proinflammatory cytokines; inhibits expression of antibodies, transferrin, and MHC II in B cells; inhibits activation of monocytes and macrophages
TNF-α	Mostly activated macrophages, but also CD4+ T cells, NK cells, PMNs, mast cells, eosinophils, neurons	Various cells	Tumor necrosis factor is a proinflammatory cytokine; induces fever (pyrogenic), apoptotic cell death; production induced by PAMPs such as LPS and in response to IL-1 and IL-6; chemokine for PMNs, stimulates macrophages
TNF-β (also known as lymphotoxin)	Lymphocytes (mostly Th1 cells)	Vascular endothelial cells	Cytokine that induces vascular endothelial cells to change surface adhesion so that phagocytic cells can bind to them; required for development of Peyer's patches

macrophages metabolize the amino acid arginine into a reactive nitrogen species (nitric oxide) and stimulate inflammation, while M2 macrophages metabolize arginine into ornithine for cycling through the urea cycle to dampen the inflammatory response. M2 macrophages also release anti-inflammatory cytokines that promote repair of damaged tissue.

Dendritic cells are closely related to macrophages. Their name derives from the fact that they are covered with spiny projections that resemble the dendrites of neurons. They are instrumental in initiating and stimulating the second responder portion of the immune response, called **adaptive immunity**. DCs are found in tissues that are in contact with the external environment or the blood and, like macrophages, the resident forms of DCs found in the dermis or in various tissue sites develop from immature monocytes circulating in the blood. Dendritic cells, such as the specialized **Langerhans cells** of skin and mucosa, become activated when they recognize bacterial PAMPs and migrate through the lymphatic system to lymph nodes, where they mature and present antigens to, and stimulate the cells of, the adaptive defense system: T helper cells (Th cells), cytotoxic T cells (also called cytotoxic T lymphocytes or CTLs), and B cells. Chapter 4 will provide a more detailed description of how DCs activate T cells and B cells.

Granulocytes: Basophils, Mast Cells, and Eosinophils

Basophils, mast cells, and eosinophils are immune cells that participate in the defense against infections, but they do not act as phagocytic cells. These granulocytes contain granules loaded with **histamine**, a vasodilator that promotes blood flow in tissues, and **heparin**, an anticoagulant that prevents blood from clotting too quickly. These cells are similar in appearance and function but differ in that basophils circulate in the blood, while mast cells congregate around blood vessels in connective tissue at mucosal sites. If any foreign material is detected, these cells release the contents of the granules, causing **vasodilation**. This helps the neutrophils and monocytes, which are normally circulating in blood, to leave the bloodstream and move to the site of infection. Eosinophils act similarly, but, unlike basophils and mast cells, they are primarily responsible for targeting multicellular parasites and viral invaders rather than bacteria.

Transmigration—How Do Phagocytes Know When and Where to Go?

Resident macrophages and DCs tend to localize in specific areas of the body. By contrast, neutrophils (PMNs) and monocytes, which are produced in the bone marrow, migrate constantly through the bloodstream. Neutrophils are the most abundant, but also the shortest-lived of the phagocytic cells; monocytes are longer-lived, but not as numerous. All of these phagocytic cell types, as well as NK cells, are capable of significantly damaging host tissues. It is not easy to kill bacteria, and considerable firepower (in the form of toxic substances released from granules or lysosomes) has to be brought to bear to destroy them. When this firepower is released by the PMNs, macrophages, and NK cells into the surrounding tissue, as is inevitable once the battle with invading bacteria is mounted, human cells also become vulnerable targets. The body protects itself from the potentially toxic action of these host defense cells by keeping them in the bloodstream in a quiescent, immature state unless danger is sensed.

How do PMNs know when to leave the blood vessels and where to go? Only when infection triggers signaling pathways that alert these cells to prepare for battle do the PMNs, monocytes, and NK cells pass through the blood vessel walls and enter tissues through a process called **transmigration** (also referred to as **diapedesis** or **extravasation**) (Figure 3-2). Two groups of proinflammatory proteins alert PMNs to leave the bloodstream, and these proteins then guide the PMNs to their destination. One of these groups is the **complement system**, whose component C3a and C5a proteins are activated by contact with invading bacteria or by interaction with antibodies bound to foreign material. Once a PMN has moved out of the blood vessel, it follows a gradient of these complement components to the site where the bacteria have invaded (**chemotaxis**).

The **cytokines** and **chemokines** are the second group of proteins (Table 3-2). Cytokines and chemokines are produced by a number of human cell types, including monocytes, macrophages, PMNs, DCs, endothelial cells, fibroblasts, and cells of the adaptive immune response (T cells and B cells). The cells of the adaptive immune system and their activities will be described in the next chapter. Complement components and cytokines/chemokines work together to orchestrate the immune response to an infection by directing the phagocytes and cytotoxic cells to the infected area and activating them through a process that is described in greater detail later in this chapter. Chemokines, like certain complement components, guide phagocytic cells to the site where an infection is occurring. Chemokines may also act as antimicrobial peptides that kill bacteria near the site of infection. Cytokines mediate the **inflammatory response** (redness, swelling, pain, fever) to invading microbes and other types of tissue damage.

When bacteria enter tissue, cytokines also stimulate neutrophils to become more actively phagocytic as they leave the bloodstream and migrate to an infected area but, unlike monocytes, PMNs do not develop into a different type of cell. During an infection, the transmigration process is accelerated. To compensate for this loss of PMNs from the bloodstream, the release of PMNs from bone marrow into the bloodstream is markedly increased. So, although passage of these phagocytic cells from the bloodstream into tissue is increased, the net effect is a higher concentration of innate defense cells in the blood. A high level of PMNs in blood is

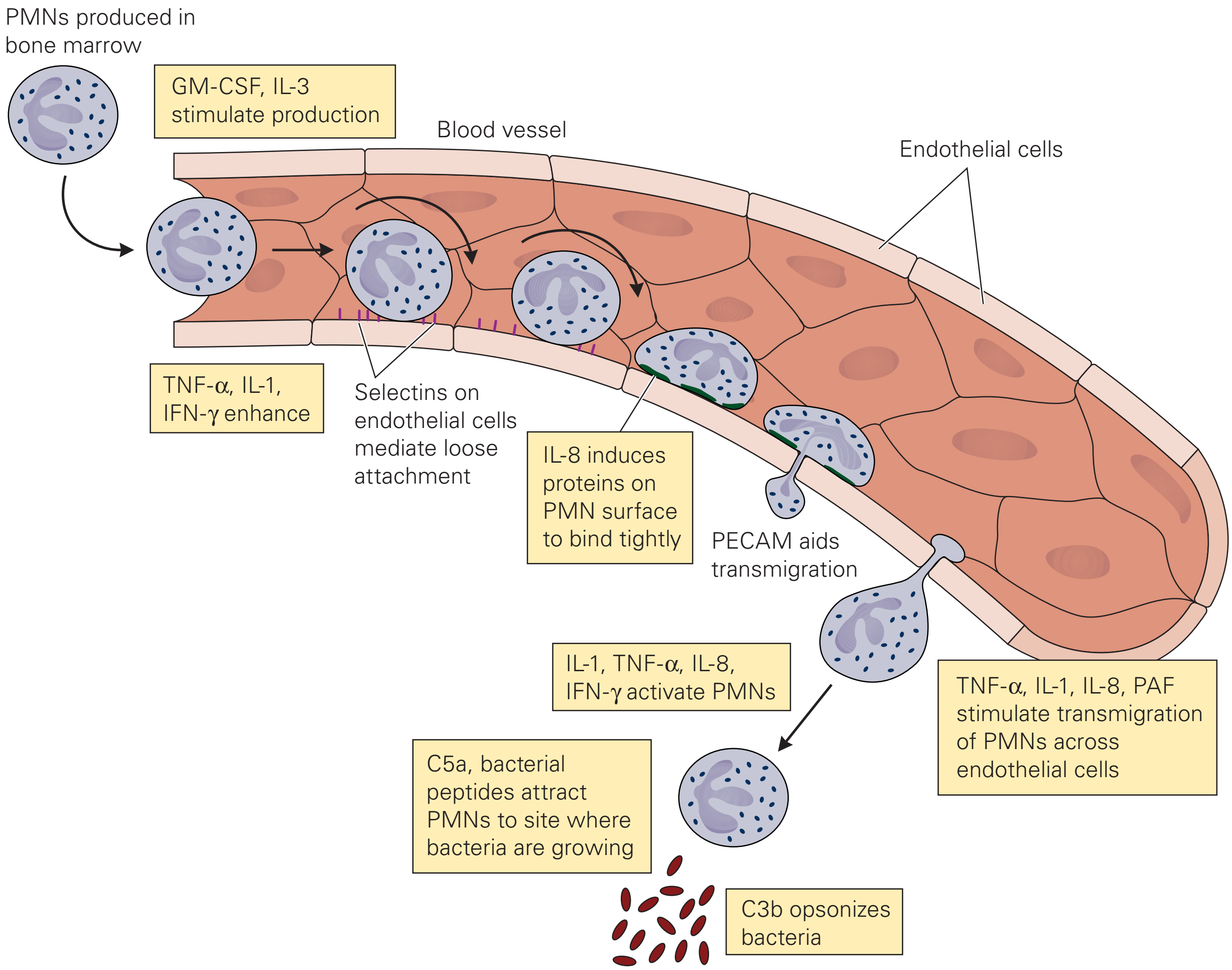

PMNs produced in bone marrow
GM-CSF, IL-3 stimulate production
Blood vessel
Endothelial cells
TNF-α, IL-1, IFN-γ enhance
Selectins on endothelial cells mediate loose attachment
IL-8 induces proteins on PMN surface to bind tightly
PECAM aids transmigration
IL-1, TNF-α, IL-8, IFN-γ activate PMNs
TNF-α, IL-1, IL-8, PAF stimulate transmigration of PMNs across endothelial cells
C5a, bacterial peptides attract PMNs to site where bacteria are growing
C3b opsonizes bacteria

Figure 3-2. Roles of various cytokines and chemokines in directing the exit of neutrophils (PMNs) from the bloodstream at infection sites. Initially, new proteins are expressed on the surfaces of PMNs and endothelial cells, permitting a loose reversible binding. This gives PMNs a rolling motility as they flow through the blood vessel. Other cytokines cause changes in the cell surfaces resulting in tighter binding. The PMNs stop moving, flatten against the vessel wall, and force themselves across the endothelial wall. PMNs then move chemotactically along a C5a gradient. PAF, platelet-activating factor; PECAM, platelet-endothelial cell adhesion molecule.

a useful diagnostic indicator of infection. During an infection, PMNs are so rapidly produced in bone marrow and dumped into the bloodstream that the immature forms of PMNs (called "**bands**" because their nuclei look like bands) are seen in the blood. The presence of bands is a clear diagnostic sign of acute infection.

Natural Killer (NK) Cells

The role of NK cells is to complement the activities of PMNs by killing human cells that are altered due to infection. Once thought to be involved primarily in controlling viral infections, NK cells are now known to be important in controlling infections by bacteria that invade and live within human cells in an attempt to evade the immune system. Unlike PMNs and other phagocytic cells, NK cells do not ingest their target, yet their mode of killing resembles that of phagocytes in many respects. Like phagocytic cells, NK cells are produced in bone marrow and circulate in the bloodstream. Also, they store their toxic substances in granules. Binding to an infected human target cell stimulates release of these granules. Instead of ingesting a bacterium or infected cell, the NK cells bombard infected cells with the toxic contents of their granules.

The granule proteins of NK cells are not the same as those of the lysosomal granules of macrophages and PMNs, but they have some similar functions. In chapter 4, we will see that these granules are very similar to those produced by cytotoxic T cells (CTLs). NK cell granules contain a protein called **perforin** that inserts into the membrane of an infected host cell and causes channels to form. These channels allow

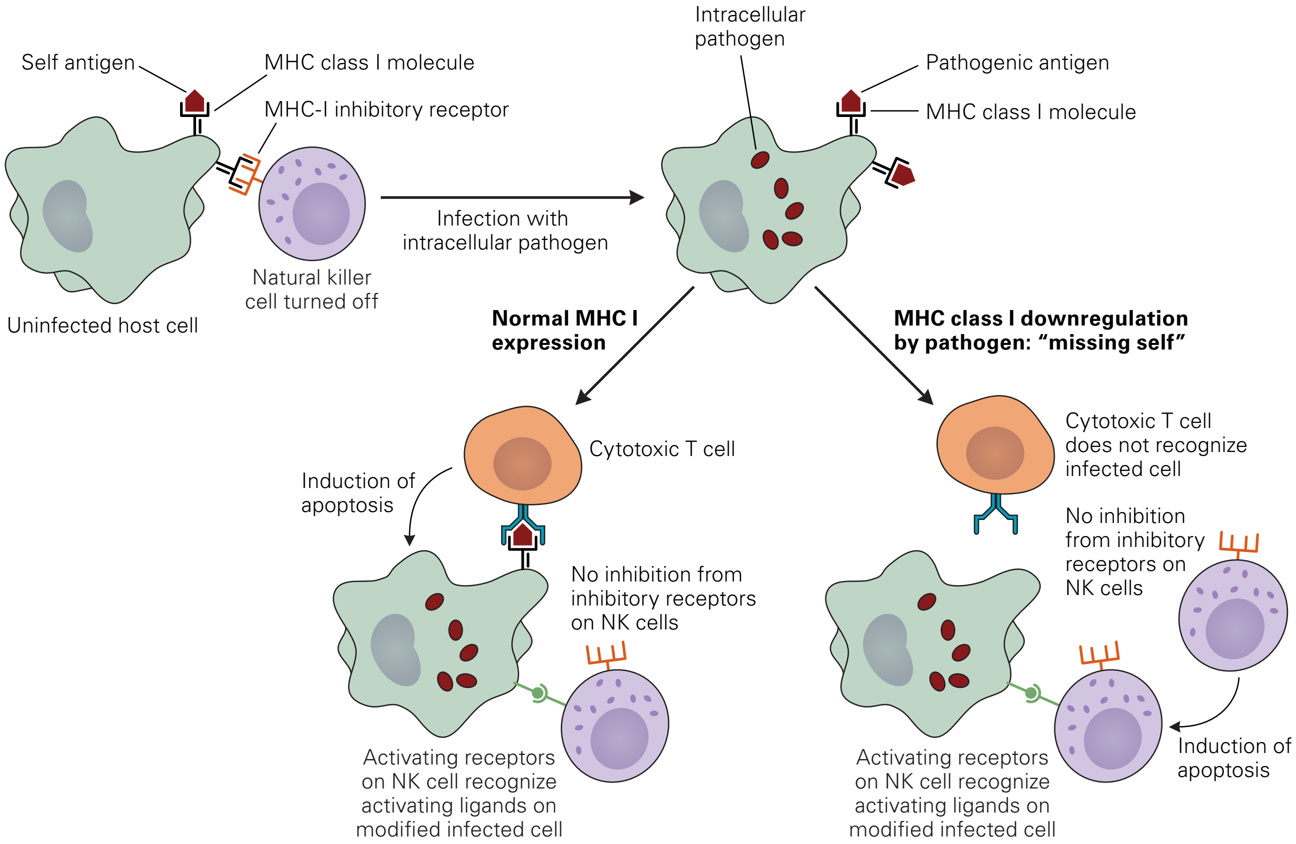

Self antigen
MHC class I molecule
MHC-I inhibitory receptor
Uninfected host cell
Natural killer cell turned off
Infection with intracellular pathogen
Intracellular pathogen
Pathogenic antigen
MHC class I molecule
Normal MHC I expression
MHC class I downregulation by pathogen: "missing self"
Cytotoxic T cell
Induction of apoptosis
No inhibition from inhibitory receptors on NK cells
Activating receptors on NK cell recognize activating ligands on modified infected cell
Cytotoxic T cell does not recognize infected cell
No inhibition from inhibitory receptors on NK cells
Activating receptors on NK cell recognize activating ligands on modified infected cell
Induction of apoptosis

Figure 3-3. Schematic diagram indicating the complementary activities of natural killer (NK) cells and cytotoxic T cells (CTLs) in killing infected host cells. Host cells that are not infected with intracellular bacteria display self-antigens through MHC I on their surfaces. However, once they become infected, their surface molecules are altered. NK cells have inhibitory receptors on their surfaces that, in the presence of MHC I bound with self-antigen, block the activation of NK cells and prevent killing of the target host cell. CTLs (described in detail in chapter 4) recognize infected cells through those complexes and subsequently kill the infected cells through release of granule contents (perforin and granzymes) that lead to induction of apoptosis. Since intracellular pathogens can cause infected cells to express far fewer MHC I molecules on their surfaces than normal cells, NK cells complement this CTL process by recognizing and killing cells that do not have MHC I complexes on their surfaces. In these cases, the inhibitory receptors do not bind MHC I and do not block the activation of the NK cells, that then proceed to release their granules and induce apoptosis in the infected host cell.

other granule proteins, a set of proteases called **granzymes**, to enter the target host cell. NK cells also secrete α-defensins that kill bacterial cells by disrupting their membranes. One effect of this assault is to force the infected host cell to initiate **apoptosis** (**programmed cell death**), a process by which the infected cell kills itself. Killing the infected host cell through apoptosis is quite different than killing through cell lysis, which can result in the release of intracellular microbes. Instead, the apoptotic process is highly regulated and controlled, producing smaller parts of the cell (called **apoptotic bodies**) that bleb off and are engulfed by nearby phagocytic cells before the contents can be released into the extracellular medium.

How do NK cells recognize and kill infected cells? As with many responses in the immune system, multiple signals are sensed to direct NK cells to kill infected cells, while sparing normal cells. NK cells express two types of surface receptors, activating receptors and inhibitory receptors, and they use an opposing-signals mechanism to identify infected cells (Figure 3-3). When NK cells encounter normal or infected cells, **activating ligands** on the surface of the target cell stimulate **activating receptors** on the surface of the NK cell. If left unchecked, this response will lead to activation of the NK cell, release of cytokines (mainly interferon gamma [IFN-γ]), and release of contents (granzymes and perforin) from granules, thereby killing the encountered cell. However, for uninfected host cells the activation of NK cells is counterbalanced by the presence of inhibitory receptors.

All normal, healthy (uninfected) cells express **class I major histocompatibility complex** (**MHC I**) proteins on their surfaces, which will be discussed in more detail in the next chapter on the adaptive immune response. NK cells can distinguish between uninfected and infected cells based on the presence or absence of MHC I, respectively. The complex of MHC I bound with a **self antigen** (small peptides that are derived from the host cell and not from a foreign invading pathogen) is recognized by an **MHC I-specific inhibitory receptor** on the NK cell surface, which then sends a signal inside the NK cell that halts activation of the cytotoxic response (release of granules) stimulated by the activating ligand-receptor interactions.

By contrast, infected cells often express much less MHC I on the surface than normal cells do, and those MHC I molecules that are on the surface are more likely to have foreign antigens (rather than self antigens) bound. Complexes of MHC I bound to foreign antigens are recognized by CTLs of the adaptive immune system (see chapter 4). The absence of self antigen-bound MHC I on the surface means that the NK cell activation response is not blocked by its inhibitory receptors, and this allows the NK cell to proceed with its attack on the infected cell.

The Lymphatic System

Lymph, the fluid that moves through the **lymphatic system,** is also monitored and protected by phagocytes. Lymphatic vessels are tubes comprised of overlapping **endothelial cells**. These tubes are organized into a network similar in complexity to the circulatory system that carries blood to all parts of the body (Figure 3-4). The role of the lymphatic system is to prevent excess buildup of fluid in tissues and to recycle blood proteins. Under healthy conditions, blood fluids and proteins leak from blood vessels in the capillary beds to feed cells, donate oxygen, and remove carbon dioxide. These fluids also provide protective blood proteins such as complement components (see below), cytokines, and chemokines. Blood vessels, however, do not readily reabsorb the fluids that leak from them. Rather, these fluids are taken care of by the lymphatic vessels. Lymphatic endothelial cells are tethered to the muscle bed, so when the level of fluids in an area becomes elevated and pressure causes muscle cells to separate, the overlapping endothelial cells of the lymphatic vessels are pulled apart. This opening allows fluid from the surrounding area to enter the lymphatic vessels, where it is then returned to central holding areas such as the thoracic duct and eventually returned to the bloodstream.

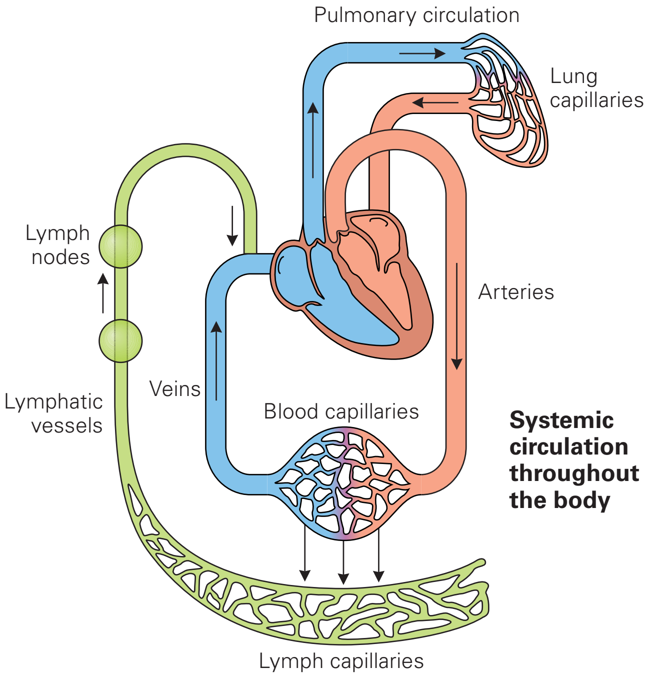


Figure 3-4. The lymphatic system.

The inflammatory response to bacterial infection creates a buildup of fluid in tissue, leading to opening of the lymphatic vessels. We experience the accumulation of blood fluids as swelling and tightness that appears around infected wounds or sites of infection. In these situations, bacteria can enter the lymphatic vessels. In order to prevent these bacteria from spreading throughout the body, the lymph must first be cleansed of these bacteria before it reenters the bloodstream. This task is accomplished by **lymph nodes** located at strategic points along the lymphatic vessels.

Lymph nodes are major sites distributed throughout the body that are linked to lymphatic vessels and contain macrophages, DCs, T cells, and B cells. Analogous to how the liver and kidneys filter toxic substances from circulation, the lymph nodes function to filter foreign entities, such as pathogens. The spleen and tonsils are other large lymphoid tissues that function similarly to lymph nodes but filter blood instead of lymph. In chapter 4, we will also learn that macrophages and DCs in the lymph nodes act as antigen-presenting cells and stimulatory cells that potentiate the adaptive defense response against bacteria. Thus, the lymph nodes not only serve to sterilize lymph but also act as sites where the adaptive defense system is alerted.

Bacteria that enter a lymph node are usually killed by the macrophages and DCs, but there are bacteria that are able to evade this fate. Indeed, some of the most dangerous pathogens are those that can survive and multiply in the lymph nodes. An example of such a pathogen is *Yersinia pestis*, the cause of **bubonic plague** (also known as the Black Death). *Y. pestis* growing in lymph nodes creates an inflammatory response so intense that it causes the lymph nodes to become grossly distended (swollen), producing the so-called **buboes** that give bubonic plague its name. A less pronounced, but still detectable, enlargement or swelling of lymph nodes (**lymphadenopathy**) likewise occurs during many types of bacterial infections and serves as a diagnostic sign of infection.

How Phagocytes Recognize and Respond to Bacteria

For many years, the interaction between PMNs and invading bacteria remained mysterious. With the discovery of PAMPs and their host cell receptors, this interaction has been a focus of intense research in recent years. Much of this research has focused on components of Gram-negative bacteria, such as the cell surface component **lipopolysaccharide (LPS)** (see Box 1-4) and **flagellin**, a protein involved in bacterial cell motility. Gram-positive bacteria also trigger the same type of PMN activation through **lipoteichoic acid (LTA)**, the anionic polymer found in the cell walls of these bacteria (see Box 1-4). **Peptidoglycan (PG)** fragments from both Gram-positive and Gram-negative bacteria, as well as certain types of DNA (e.g., **CpG-rich DNA**) and RNA, can also act as triggers.

PAMPs were discovered in an unusual way. Scientists studying the fruit fly, *Drosophila melanogaster*, identified a transmembrane receptor protein called **Toll** that was required for resistance to fungal infections. Oddly enough, only one of the Toll receptors in *Drosophila* is linked to a defense against infection; the others are involved in various aspects of fly development. Shortly thereafter the mammalian equivalent of Toll was discovered—hence the name "**Toll-like receptor (TLR)**,"—and shown to be important for immunity. To date, thirteen mammalian TLR **paralogs** (genes found in different species that are traceable to a shared ancestry) have been described and have been shown to modulate cellular responses to pathogens. Most mammals have ten to thirteen different TLRs (humans have ten functional TLRs).

Although there are many different members of the TLR family, one common set of them is evolutionarily conserved in a wide range of organisms. This common set of diverse TLRs may reflect the fact that although all Gram-negative and Gram-positive bacteria have surface "signatures" in common, there are also numerous differences. The different TLRs in various combinations (through homo- and heterodimerization) and coupling with different downstream adaptor molecule combinations provide the flexibility to respond to differences between invading bacteria without responding specifically to each particular bacterium, as the antibody-based adaptive immune system does. For example, TLR4 is a receptor for LPS from most Gram-negative bacteria, but sometimes a complex of TLR1/TLR2 or TLR2/TLR6 is the receptor for certain pathogens that have modified LPS structures or surface lipoproteins. TLR9 responds to DNA containing stretches of unmethylated CpG sequences, which is not usually found in mammalian DNA. Double-stranded RNA activates TLR3 and TLR8, while single-stranded RNA activates TLR7, and bacterial flagellin activates TLR5. The ligand for TLR10 is not yet known. Mice have a TLR11 that recognizes parasites and uropathogenic *Escherichia coli*, but in humans the gene encoding TLR11 has multiple stop codons that prevent expression of a functional protein.

Figure 3-5 summarizes the different TLRs in humans and mice and where they are localized in the cell, as well as the signaling pathways that they activate. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are exposed on the surface of the phagocytic cell, whereas TLR3, TLR7, TLR8, and TLR9 reside within endosomes. Each TLR contains a leucine-rich repeat (LRR) domain that is extracellular for the surface TLRs or faces into the lumen of the vesicle for the endosomal TLRs. The LRR domain binds the PAMP ligand(s) and induces dimerization. The intracellular domain of each TLR has a conserved region of amino acid homology with interleukin-1 receptors (IL-1Rs), referred to as the Toll/IL-1R (TIR) domain. The TIR domain conveys the signal detected by the LRR domain binding to a PAMP ligand to the interior of the phagocytic cell through a signal transduction cascade mediated through adaptor proteins and kinases, including the myeloid differentiation primary response gene 88 protein (MyD88), IL-1R-associated kinases (IRAKs), transforming growth factor β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1), TAB2, tumor necrosis factor receptor-associated factor 6 (TRAF6), and others. In all cases, activation of these signaling pathways leads to expression of inflammatory genes and induction of phagocytic functions, such as maturation and secretion of cytokines and stimulation of the oxidative burst that kills opsonized bacteria.

The process by which bacterial components (PAMPs) trigger cytokine release through TLR signaling is best understood in the case of LPS (see Box 1-4). LPS that is released from outer membranes due to bacterial lysis binds to **LPS-binding protein** (**LBP**), a blood protein produced by the liver, and is delivered to **CD14**, a protein receptor on the surface of macrophages and other cytokine-producing cells (Figure 3-6). LPS is then transferred to the transmembrane signaling receptor TLR4 and its accessory protein **MD2**. This TLR4/LBP/CD14/MD2 complex triggers a cellular signal transduction pathway that leads to activation of the transcription factor NF-κB, which then moves to the nucleus and induces inflammatory cytokine gene expression.

The host cell also has a PAMP detection system for intracellular pathogens, called **NOD-like receptors (NLRs)**. Humans have twenty-two different NLR proteins that sense a variety of PAMPs, including cell wall components of intracellular bacteria, toxins, and host-derived ligands (such as damaged membrane components). NLRs consist of three domains: a variable N-terminal domain with different sensor-like functions (depending on the type of NLR); a central nucleotide-binding domain that mediates ATP-dependent auto-oligomerization (called a NACHT domain); and a C-terminal LRR domain that functions analogously to that of TLRs to detect bacterial-derived PAMPs inside host cells (peptidoglycan components in the case of NOD1 and NOD2, flagellin in the case of NAIP and NLRC4, and anthrax toxin in the case of NLRP1). Activation of most NLRs leads to NLR oligomerization and formation of the **inflammasome**, a complex of multiple signaling (NLR, caspase-1) and adaptor (ASC) proteins that mediate oligomerization and self-cleavage of pro-caspase-1, a **zymogen** (a proenzyme that is inactive until a proteolytic cleavage event converts it into its active form) (Figure 3-7).

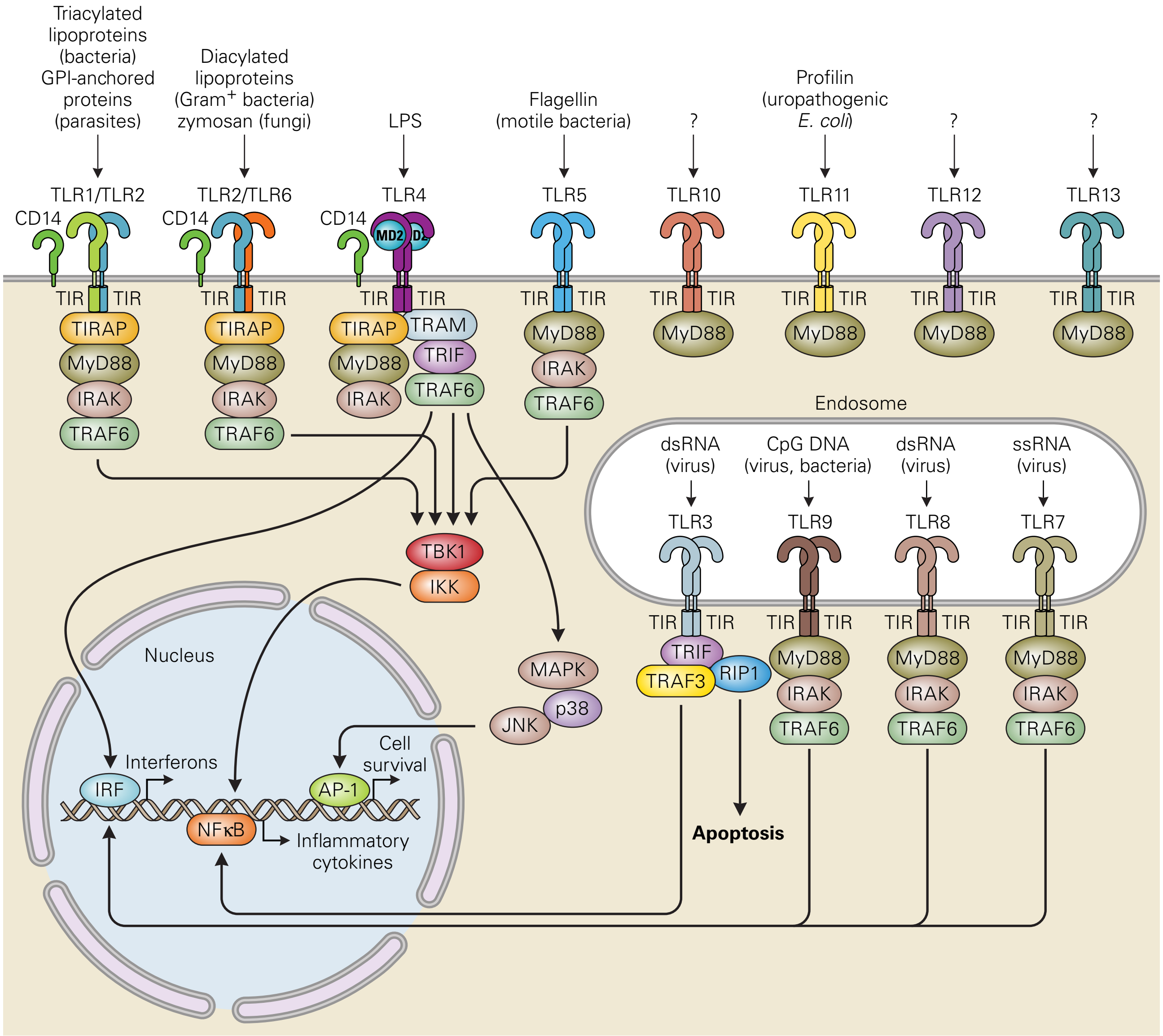

Triacylated lipoproteins (bacteria) GPI-anchored proteins (parasites)
Diacylated lipoproteins (Gram$^+$ bacteria) zymosan (fungi)
LPS
Flagellin (motile bacteria)
?
Profilin (uropathogenic E. coli)
?
?
TLR1/TLR2
TLR2/TLR6
TLR4
TLR5
TLR10
TLR11
TLR12
TLR13
CD14
CD14
CD14
MD2
D2
TIR
TIRAP
TRAM
TRIF
MyD88
IRAK
TRAF6
Endosome
dsRNA (virus)
CpG DNA (virus, bacteria)
dsRNA (virus)
ssRNA (virus)
TLR3
TLR9
TLR8
TLR7
TBK1
IKK
Nucleus
MAPK
p38
JNK
RIP1
TRAF3
Cell survival
Interferons
IRF
AP-1
NFκB
Inflammatory cytokines
Apoptosis

Figure 3-5. The Toll-like receptor (TLR) family and their signaling pathways in humans and mice. TLRs mediate the activation of the innate immune system through recognition of pathogen-associated molecular patterns (PAMPs). TLR1, TLR2, and TLR6 form heterodimers on the plasma membrane that bind to microbial membrane components. TLR4 forms homodimers that bind with MD2, CD14, and LPS bound LBP on the plasma membrane (see Figure 3-6). TLR5 forms homodimers on the plasma membrane that bind bacterial flagellin. TLR3, TLR7, TLR8, and TLR9 form homodimers inside endocytic vesicles that recognize various forms of nucleic acids. The ligands for TLR10, TRL12, and TLR13 are unknown. Humans have a mutated TLR11 gene that does not express protein, and do not have genes for TLR12 or TLR13. In mice TLR11 is functional and recognizes parasites and uropathogenic bacteria. TLRs activate downstream signaling pathways that lead to expression of proinflammatory cytokines through various adaptor proteins (MyD88, TIRAP, TRAM, TRIF, IRAK, TRAF6, TRAF3, and RIP-1) that interact with their intracellular TIR domains, which have homology with the receptors for IL-1 and IL-18.

Caspase-1 signaling pathways lead to a form of programmed cell death, called **pyroptosis**, where cells produce cytokines, swell, and burst. Caspase-1 proteolytic activity leads to maturation of proinflammatory cytokines, especially interleukin-1β (IL-1β). Production of these cytokines leads to inflammatory responses in macrophages, such as Th1 responses and release of IFN-γ, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8), and activation of NK cells.

The best-described members of the NLR family are the **NOD1** and **NOD2** proteins, which have an N-terminal caspase activation and recruitment domain (CARD). Human NOD1 detects a part of the peptidoglycan derived from degradation of the Gram-negative cell wall, whereas NOD2

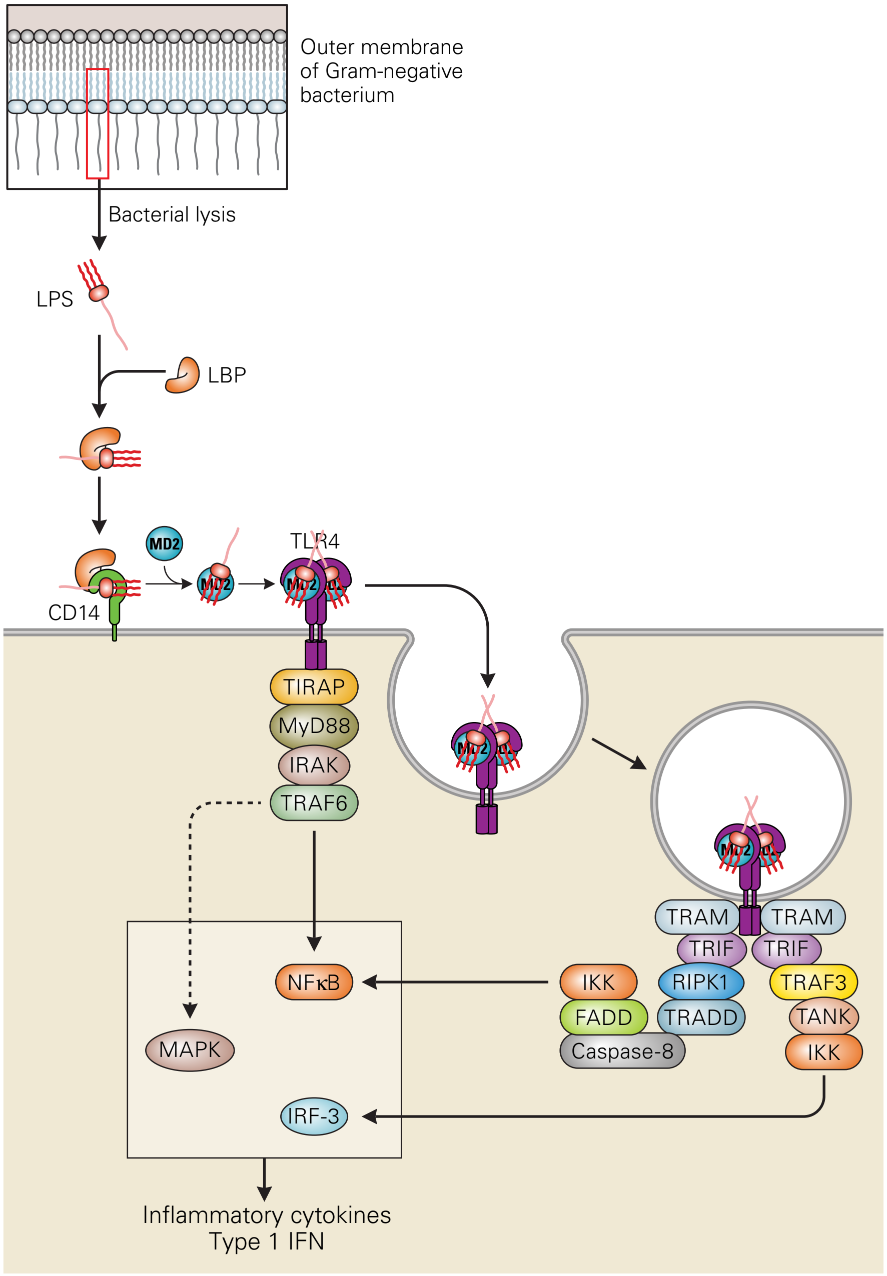

Outer membrane
of Gram-negative
bacterium
Bacterial lysis
LPS
LBP
CD14
MD2
TLR4
TIRAP
MyD88
IRAK
TRAF6
NFκB
MAPK
IRF-3
TRAM
TRAM
TRIF
TRIF
IKK
RIPK1
TRAF3
FADD
TRADD
TANK
Caspase-8
IKK
Inflammatory cytokines
Type 1 IFN

Figure 3-6. Cellular recognition of LPS through Toll-like receptor signaling.

detects a degradation product derived from Gram-positive peptidoglycan. While NOD1 is expressed by many cell types, NOD2 is expressed primarily in macrophages, DCs, Paneth cells, keratinocytes, and mucosal epithelial cells. Interestingly, NOD1 and NOD2 are unlike other NRLs in that they do not appear to form inflammasomes that directly activate caspase-1, but rather they form their own signaling complex, called a **nodosome**. The nodosome is directed to the site of bacterial entry into the cell, where it also interacts with the receptor-interacting serine/threonine-protein kinase 2 (Rip2/RIPK2) through the CARD domains. This is followed by recruitment of a number of other signaling molecules and potent activation of the transcription factor NF-κB, which is transported into the nucleus, where it induces expression of IL-1β, IL-18, and other proinflammatory genes.

In addition to sending out cytokine signals to alert the immune system of infection by pathogens, host cells that are severely damaged as a result of pathogen invasion also produce and release other endogenous host cell molecules, called **alarmins**. Alarmins are released into the extracellular

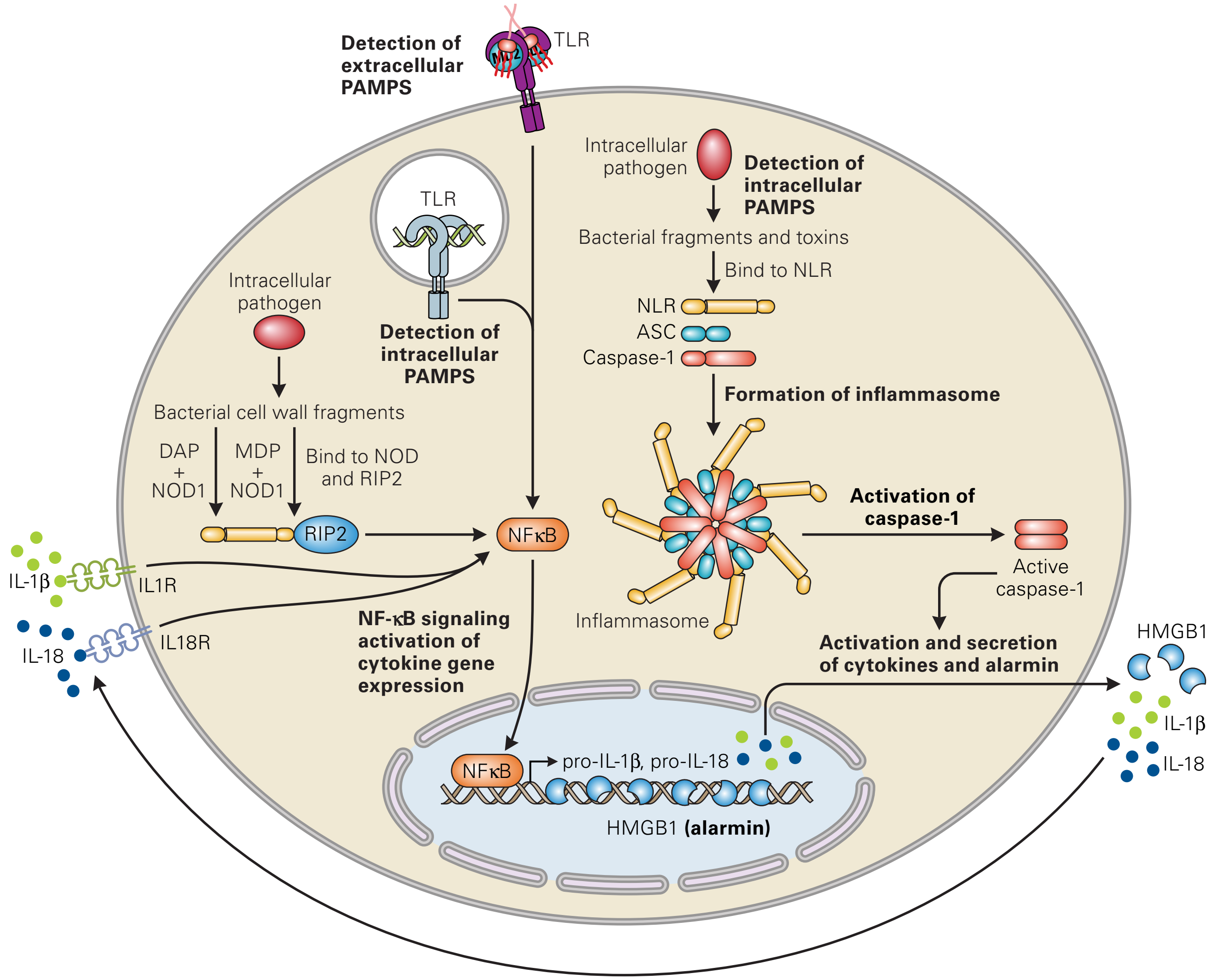

Detection of extracellular PAMPS
TLR
MD2
TLR
Detection of intracellular PAMPS
Intracellular pathogen
Bacterial cell wall fragments
DAP + NOD1
MDP + NOD1
Bind to NOD and RIP2
RIP2
NFκB
Intracellular pathogen
Detection of intracellular PAMPS
Bacterial fragments and toxins
Bind to NLR
NLR
ASC
Caspase-1
Formation of inflammasome
Activation of caspase-1
Active caspase-1
Inflammasome
Activation and secretion of cytokines and alarmin
HMGB1
IL-1β
IL-18
IL-1β
IL1R
IL-18
IL18R
NF-κB signaling activation of cytokine gene expression
NFκB
pro-IL-1β, pro-IL-18
HMGB1 (alarmin)

Figure 3-7. NOD-like receptor (NLR) sensing of intracellular pathogens. PAMP activation of TLRs and binding of the proinflammatory cytokines (IL-1β and IL-18) to their cognate receptors stimulate NF-κB signaling that leads to expression of pro-IL-1β and pro-IL-18. Infection with intracellular pathogens induces assembly of the NLRs, adaptor protein (ASC), and caspase-1 into inflammasome complexes, which lead to caspase-1 activation and subsequent cleavage and secretion of IL-1β and IL-18, as well as release of alarmin (HMGB1) and induction of pyroptosis. Unlike other NLRs, NOD1 binds the bacterial peptidoglycan derived D-Glu-meso-diaminopimelic acid (DAP), while NOD2 binds muramyl dipeptide (MDP) to form a nodosome complex, comprised of the ligand-bound NOD and a serine/threonine kinase adaptor protein, Rip2 (also called RIPK2), which in turn activates NF-κB. Adapted from Lamkanfi M, Dixit VM. 2011. *J Immunol* **187**:597–602, with permission.

medium by infected cells that are lysed or die by nonapoptotic mechanisms. One such alarmin is the **high mobility group box 1 (HMGB1) protein,** a nuclear protein that binds to nucleosomes and promotes DNA bending. Cells that are undergoing apoptosis modify their chromatin such that HMGB1 remains tightly bound to the DNA and not released, whereas cells that are dying by necrosis or lysis release HMGB1 into the extracellular medium. In response to PAMP detection and inflammatory stimulation, cells can also actively secrete HMGB1, which then serves as a chemokine to recruit phagocytes to the site of infection.

How Phagocytes Kill Bacteria

Oxidative Burst in Phagolysosomes

The steps involved in the killing of a bacterium by a phagocyte are shown in Figure 3-8. The phagocyte first forms pseudopods that engulf the bacterium. **Phagocytosis** (engulfment) requires dynamic rearrangements of actin, a major component of the eukaryotic cytoskeleton. After engulfment, the bacterium is encased in an endocytic vesicle called a **phagosome**. The phagosomal membrane contains ATPases that pump protons into the phagosome interior, reducing

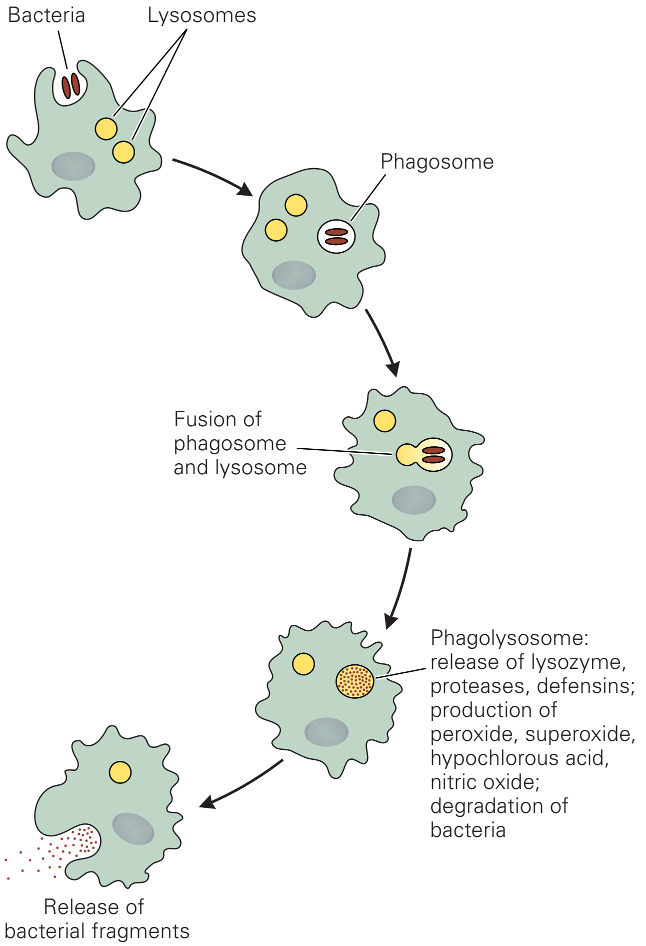

Bacteria
Lysosomes
Phagosome
Fusion of phagosome and lysosome
Phagolysosome: release of lysozyme, proteases, defensins; production of peroxide, superoxide, hypochlorous acid, nitric oxide; degradation of bacteria
Release of bacterial fragments

Figure 3-8. Steps in ingestion and killing of bacteria by phagocytes. Bacteria are first engulfed by endocytosis into a phagosome. Fusion of phagosomes and lysosomes releases toxic enzymes and proteins that kill most bacteria. Debris from dead bacteria is then released by exocytosis.

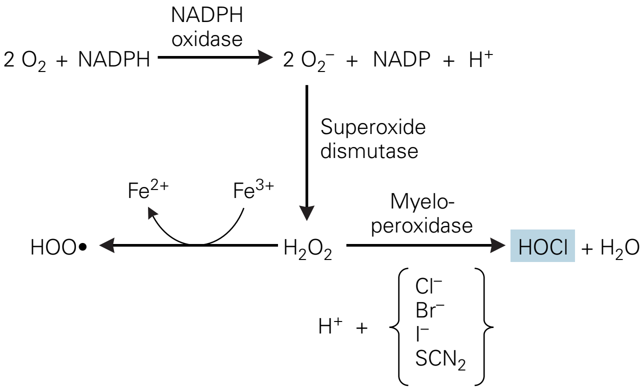


Figure 3-9. Oxidative burst in the phagolysosome. The phagosome contains two unique enzymes: lysosomal myeloperoxidase and phagosomal membrane-bound NADPH oxidase. Superoxide dismutase converts the superoxide radical (O_2^-) generated by NADPH oxidase into hydrogen peroxide (H_2O_2). Myeloperoxidase then catalyzes the reaction of hydrogen peroxide with chloride (Cl^-), thiocyanate (SCN^-), or other halide ions to form hypochlorite (^-OCl), the active ingredient in bleach, or hypothiocyanite ($OSCN^-$), both of which are extremely toxic to bacteria. Under these oxidative conditions iron ions exist predominantly in the oxidized ferric (Fe^{3+}) state, and this free Fe^{3+} plus hydrogen peroxide forms hydroperoxyl radical (HOO•) via a nonenzymatic Fenton reaction, that can also damage macromolecules such as proteins and DNA.

the internal acidity to as low as pH 4.5. Phagocytes also carry antibacterial proteins that are as toxic to the phagocytes and surrounding tissue cells as they are to their bacterial targets. Accordingly, these toxic proteins are stored in an inactive form in **lysosomes**. Fusion of a lysosome with a phagosome to form a **phagolysosome** releases the lysosomal proteins into the phagolysosome interior, where they are activated by the low pH of the phagolysosome interior.

Phagocytes possess two general types of bacterial killing mechanisms mediated by the fusion of phagosomes and lysosomes: non-oxidative and oxidative. Lysosomal vesicles contain various macromolecule-degrading enzymes (proteases, lipases, glycosidases, hydrolases, nucleases, and lysozyme) that destroy bacterial components and mediate **nonoxidative killing**. Degrading enzymes, such as proteases, phospholipases, glycosidases, hydrolases, and lysozyme, destroy surface and membrane components of the bacteria. Nucleases hydrolyze bacterial DNA and RNA that are released during lysis. Other lysosomal proteins, such as **defensins**, antimicrobial peptides, and other membrane permeabilizers, insert into bacterial membranes and create pores that permeabilize the membrane, kill the bacteria, and allow the bacterial cytoplasmic components to leak into the surrounding environment.

Oxidative killing occurs through formation of toxic reactive oxygen and reactive nitrogen species. Lysosomes have another type of lysosomal protein, **myeloperoxidase**, that produces reactive forms of oxygen that are toxic to many bacteria (similar to lactoperoxidase). The generation of toxic forms of oxygen by phagocytes is called the **oxidative (or respiratory) burst**. Myeloperoxidase is only activated when it is brought into contact with an **NADPH oxidase**, which is located in the phagosomal membrane, and when the resulting complex is then exposed to the low pH of the phagolysosome interior. During an infection, cytokines stimulate increased production of these lysosomal enzymes, thus increasing the killing potential of the oxidative burst.

The reaction catalyzed by the myeloperoxidase complex has three steps (Figure 3-9). First, NADPH oxidase generates a **superoxide radical**: $\text{NADPH} + 2\text{O}_2 \longrightarrow 2\text{O}_2^-$ (superoxide radical) + $\text{NADP}^+ + \text{H}^+$. The superoxide radical is extremely reactive and is readily converted into hydrogen peroxide (H_2O_2) by **superoxide dismutase**. Myeloperoxidase catalyzes the reaction of hydrogen peroxide with chloride (Cl^-)

or thiocyanate (SCN^-) ions (as well as bromide or iodide ions) to form **hypochlorite** (^-OCl, the active ingredient in bleach) or **hypothiocyanite** (^-OSCN), respectively. These reactive oxygen molecules are toxic to bacteria because they inactivate essential bacterial surface proteins through oxidization of sulfur-containing amino acids and molecules, such as cysteine, methionine, and glutathione. For example, oxidation of the thiol groups (-SH) of the amino acid side chain of cysteine in proteins results in formation of sulfenic acid, sulfonic acid, or disulfide linkages.

Under these oxidative conditions iron ions exist predominantly in the oxidized ferric (Fe^{3+}) state. Inside the body, Fe^{3+} ions are bound to iron-binding proteins, such as transferrin or lactoferrin, but in phagolysosomes the low pH environment causes the release of Fe^{3+} from the proteins. The freed Fe^{3+} ion plus hydrogen peroxide (H_2O_2) forms hydroperoxyl radical (HOO•) via the nonenzymatic **Fenton reaction**, which can also damage DNA.

Human monocytes and macrophages also produce another very simple but powerful antimicrobial compound, **nitric oxide** (**NO**). NO is toxic in its own right, attacking bacterial metalloenzymes, proteins, and DNA. Additionally, it can combine with superoxide to form **peroxynitrite** (**$OONO^-$**), a very reactive molecule that oxidizes amino acids and is toxic to both bacteria and human cells. Synergistic reactions between NO and superoxide during the oxidative burst may help make the burst more toxic for bacteria. NO may also serve as a signaling molecule to regulate the functions of phagocytic cells, as well as other adaptive immune cells. NO has been implicated in so many areas of human and animal physiology that the journal *Science* chose it as the molecule of the year in 1992.

During an infection, cytokines induce NO synthesis by many human cell types. NO production is indicated by the presence of the stable end products of its oxidation, nitrite and nitrate, in the blood. NO may also contribute to some of the symptoms of disease, including vascular collapse and tissue injury, as mice lacking the inducible NO synthetase responsible for generating NO tolerate bacterial endotoxin with fewer toxic side effects than normal mice. On the other hand, these mice are highly susceptible to infections by intracellular bacterial and protozoan pathogens, such as *Mycobacterium tuberculosis* or *Leishmania major*, which cause tuberculosis or leishmaniasis, respectively. The human pathogen *Neisseria meningitidis*, which is responsible for meningococcal disease, has at least two NO detoxification enzymes that enhance survival during nasopharyngeal colonization and during phagocytosis by human macrophages.

Autophagy—Another Pathway for the Killing of Intracellular Pathogens

Some intracellular pathogens, such as *Mycobacterium tuberculosis* and *Salmonella enterica* serovar Typhimurium, are able to escape destruction through the phagolysosomal pathway by modifying the phagosome into a specialized vacuole that does not fuse with lysosomes. Others, such as *Rickettsia conorii*, escape the phagosome and reside within the host cytosol. In such cases of intracellular pathogenesis, another cellular pathway, called **autophagy,** is employed. Autophagy normally enables the breakdown and recycling of dysfunctional cellular components, such as mitochondria, by sequestering them from the rest of the cytosolic components into a double-membrane organelle called an **autophagosome**, which then fuses with a lysosome to destroy the contents of the resulting **autophagolysosome**.

Autophagosome formation is initiated by a precursor **isolation membrane (phagophore)**, to which two ubiquitin-like conjugation complexes are recruited. One of these involves the ubiquitin-like **autophagy-related protein Atg8** (also called **LC3**) that is conjugated with a membrane phospholipid, **phosphatidylethanolamine (PE)**. LC3-PE mediates membrane tethering and expansion of the phagophore that associates with and encircles the intracellular pathogen or the pathogen-containing vesicle. The second ubiquitin-like complex is comprised of ubiquitin-like **Atg12** conjugated with the adaptor protein **Atg5** and noncovalently associated with the adaptor protein **Atg16**. The Atg12-Atg5-Atg16 complex oligomerizes and is recruited to the newly formed isolation membrane and controls elongation and eventual closure of the phagophore.

Intracellular pathogens can be targeted for degradation through autophagy (Figure 3-10). Bacteria that escape into the cytosol of the cell become ubiquitinated on the surface, which recruits adaptor proteins, such as **NDP52** or **p62**, which can bind both ubiquitinated proteins on the bacterial surface and LC3-PE attached to phagophores. Another adaptor protein is **optineurin (OPTN),** which binds LC3 and ubiquitinated bacteria when it becomes phosphorylated by the kinase TBK1, a regulator of NF-κB signaling and interferon production.

In the case of phagosomes containing invasive bacteria, such as *M. tuberculosis* or *S.* Typhimurium, autophagy is triggered by the damage to the phagosomal membrane caused by the bacterium. Recently, **galectin-8** (a β-galactoside-binding lectin) has been identified as a cytosolic detector protein that can initiate the autophagy response for invading pathogens. Galectin-8 recognizes galactoside residues in exposed, damaged membrane and recruits autophagy adaptor proteins, such as NDP52, which binds to the autophagy proteins **LC3** and **Atg12** and brings the phagophore to the altered bacterium-containing phagosome. NOD2 protein can also initiate autophagy through recruitment and activation of the autophagy protein **Atg16**, which controls elongation of new autophagosomal membrane, at the site of bacterial entry into the cell.

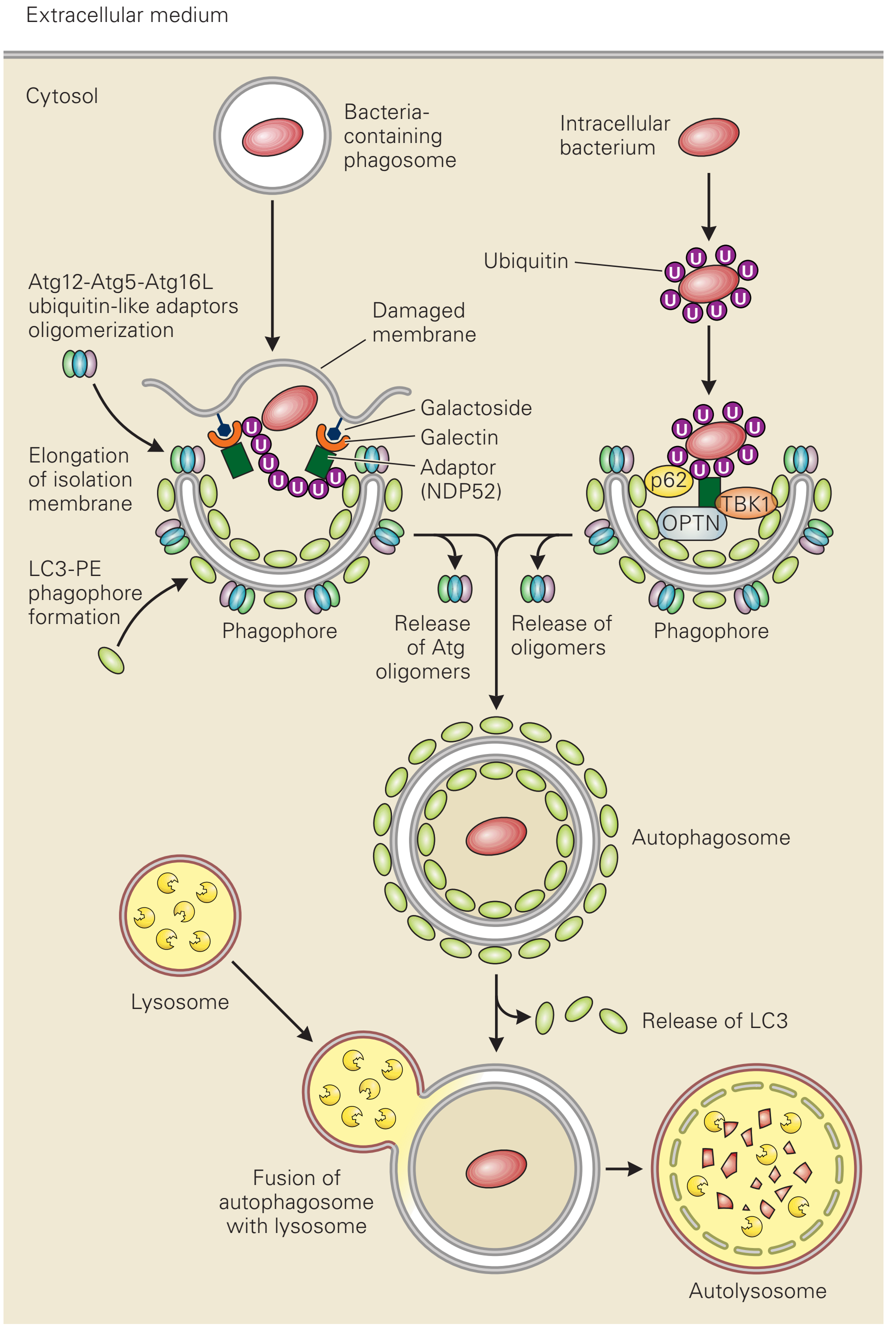

Extracellular medium
Cytosol
Bacteria-containing phagosome
Intracellular bacterium
Ubiquitin
Atg12-Atg5-Atg16L ubiquitin-like adaptors oligomerization
Damaged membrane
Galactoside
Galectin
Adaptor (NDP52)
Elongation of isolation membrane
p62
TBK1
OPTN
LC3-PE phagophore formation
Phagophore
Release of Atg oligomers
Release of oligomers
Phagophore
Autophagosome
Lysosome
Release of LC3
Fusion of autophagosome with lysosome
Autolysosome

Figure 3-10. Eliminating intracellular pathogens through autophagy. Intracellular pathogens that escape into the cytosol or invading pathogens that enter via phagosomes or reside in pathogen-specific vacuoles are targeted for lysosomal degradation by autophagy. The target cytosolic pathogen or pathogen-containing vesicle is engulfed into a specialized double-membrane organelle called an autophagosome, which then fuses with a lysosome to become an autolysosome.

The Complement Cascade

Complement Proteins

The skin and mucosa barriers, along with the phagocytic cells, are powerful defenses against bacterial invaders. There is, however, another highly complex and equally important arm of the innate defenses—the blood proteins that mark targets for phagocytic destruction and direct the inflammatory response. These **complement system** proteins are produced by the liver, circulate in the blood, and enter tissues throughout the body. Their primary purpose is to help phagocytic cells and antibodies rapidly clear pathogens from the body. Along with chemokines and cytokines, complement proteins also form an important link between the innate phagocytic defense system and the adaptive immune defenses, which will be discussed further in chapter 4.

Many of the complement proteins are zymogens, which reside throughout the body as inactive precursors. **Complement activation** occurs during infection as the complement proteins undergo a cascade of activating proteolytic cleavages, where each activated complement zymogen cleaves and activates the next complement zymogen. In terms of nomenclature, a letter “C” is used to designate the core complement components, some of which are actually multi-protein complexes. There are nine of these core components, C1 to C9. Activated proteolytic cleavage products are indicated by an “a” or “b.” In most cases, as, for example, C3, C4, and C5, “a” designates the smaller and “b” the larger of the two proteolytic products (e.g., C3a and C3b, respectively), but this rule is not followed uniformly, notably with regard to the complement component C2. Unfortunately for us, most immunologists prefer to retain the old nomenclature for C2, where the small C2 cleavage product is designated C2b and the large one C2a. We will use this designation.

Overview of Complement Pathways and Their Function

When activated through proteolytic cleavage and complex formation, the complement system performs five important interrelated functions: (1) promotion of **opsonization** (engulfment) of invading bacteria by coating them with complement components (e.g., C3b); (2) enhancement of chemotaxis of phagocytes (PMNs, monocytes, macrophages, and DCs) to attract them to the site of infection by releasing chemokines (e.g., C5a); (3) enhancement of vascular permeability to promote exudation (transmigration) of phagocytes from blood vessels into tissues at the site of infection by releasing vasodilators (e.g., C3a, C4a, and C5a) that cause degranulation (release of histamine and heparin) of basophils and mast cells; (4) promotion of **agglutination** (clustering and clumping) of invading bacteria by binding them together and increasing the efficiency of phagocytosis and clearance from the system; and (5) direct killing of many Gram-negative bacteria by poking holes in their membranes via binding of C5b to LPS and formation of the **membrane attack complex (MAC)** that consists of components C5b through C9.

Complement activation can be initiated through three pathways, as shown in Figure 3-11. The first two pathways are antibody-independent and form part of the innate response to initial infection, while the third pathway is antibody-dependent and comes into play after adaptive immunity has been launched. All three pathways lead to activation of the pivotal complement component, the protease **C3-convertase**, that cleaves and activates C3 into C3a and C3b. C3b covalently binds to the surface of pathogens, coating them and enhancing agglutination and opsonization of the pathogen by phagocytes.

Certain surface components of bacteria can trigger the complement cascade through the **alternative pathway** without the need for adaptive responses. The best-characterized complement-triggering bacterial surface molecules are LPS and LTA found in the outer and inner cell membranes of Gram-negative and Gram-positive bacteria, respectively. Complement-activating molecules of fungi, protozoa, and metazoa are not as well characterized, but they also appear to be lipid-carbohydrate complexes on the microbial cell surface. In the alternative pathway, the free hydroxyl (–OH) groups or amino (–NH_2) groups of bacterial surface components react with an activated thioester group that is present in the C3b protein to form a covalent linkage that triggers the alternative pathway by recruiting factor B to the bacterial surface and initiating formation of the C3-convertase.

A more recently discovered initiation pathway that also responds to initial infection, called the **lectin pathway**, involves multimeric proteins called **mannose-binding lectins (MBL)**, which are members of a family of PAMP-recognizing proteins called **collectins.** Collectins are calcium-dependent lectins (i.e., proteins that bind specifically to certain sugar residues in the presence of calcium). The MBLs bind mannose groups that are commonly found on the surfaces of bacteria but not on human cells. Recently, another group of PAMP-binding lectins, called **ficolins (FCNs)**, which bind specifically to N-acetylglucosamine (GlcNAc) groups, have been shown to activate the lectin pathway in a similar manner. Collectins are produced by the liver and are part of what is called the **acute phase response** (or **acute inflammatory response**) to an infection—the initial onslaught by a variety of proteins, including cytokines and iron-binding proteins, that make it difficult for bacteria to multiply. Collectins that are bound to the surface of a bacterium not only sequester the bacteria into clumps that are then eliminated from the body by phagocytic cells, but they can also activate the complement cascade to form the C3-convertase via the lectin pathway.

Finally, antibodies generated through the adaptive defense system (to be discussed in more detail in chapter 4) can also activate complement by binding to the surface of bacteria and interacting with the C1-complex. Antibodies are blood proteins produced by B cells that bind to specific molecules on the bacterial surface called antigens. Antigen-bound antibodies can bind the C1 complex to activate it, initiating the cascade that ultimately leads to formation of the C3-convertase. Thus, both the innate and adaptive defense systems can trigger the complement cascade—yet another example of a link between the innate and adaptive defenses.

Before examining each of the pathways for complement activation in detail, it is helpful to understand the roles of

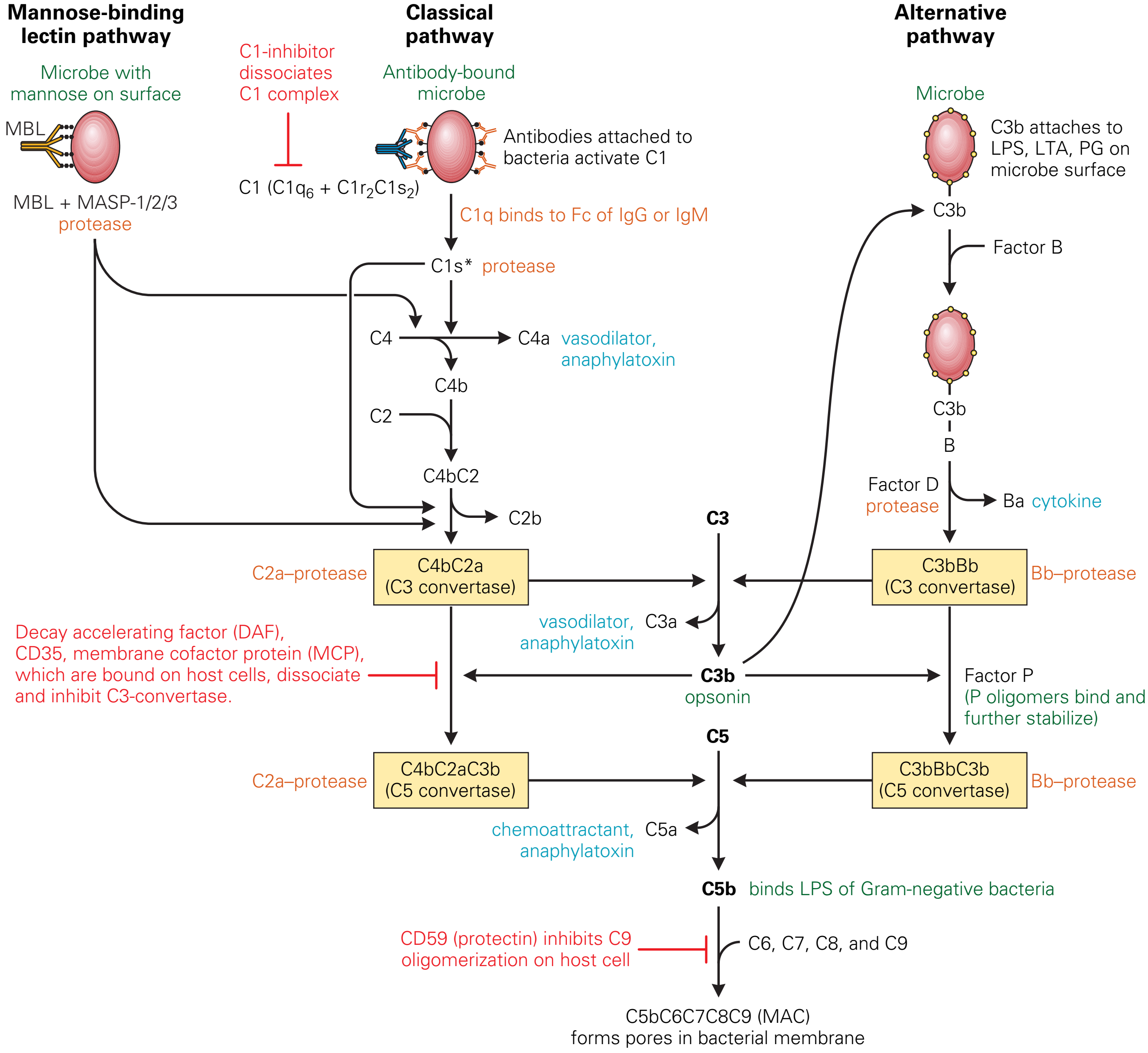

Mannose-binding lectin pathway
Microbe with mannose on surface
MBL
MBL + MASP-1/2/3 protease
C1-inhibitor dissociates C1 complex
Classical pathway
Antibody-bound microbe
Antibodies attached to bacteria activate C1
C1 ($C1q_6$ + $C1r_2C1s_2$)
C1q binds to Fc of IgG or IgM
C1s* protease
C4
C4a vasodilator, anaphylatoxin
C4b
C2
C4bC2
C2b
Alternative pathway
Microbe
C3b attaches to LPS, LTA, PG on microbe surface
C3b
Factor B
C3b
B
Factor D protease
Ba cytokine
C2a–protease
C4bC2a (C3 convertase)
C3
C3bBb (C3 convertase)
Bb–protease
vasodilator, anaphylatoxin
C3a
Decay accelerating factor (DAF), CD35, membrane cofactor protein (MCP), which are bound on host cells, dissociate and inhibit C3-convertase.
C3b
opsonin
Factor P
(P oligomers bind and further stabilize)
C5
C2a–protease
C4bC2aC3b (C5 convertase)
C3bBbC3b (C5 convertase)
Bb–protease
chemoattractant, anaphylatoxin
C5a
C5b binds LPS of Gram-negative bacteria
CD59 (protectin) inhibits C9 oligomerization on host cell
C6, C7, C8, and C9
C5bC6C7C8C9 (MAC)
forms pores in bacterial membrane

Figure 3-11. Main steps in activation of complement by the mannose-binding lectin, classical, and alternative pathways. These pathways differ only in the steps that initiate formation of C3 convertase. Important activated products are C3b (which opsonizes bacteria), C3a (which acts as a vasodilator), C5a (which acts as a vasodilator and a chemokine that attracts phagocytes to the area), and C5b-C9 (MAC [membrane attack complex], which inactivates enveloped viruses and kills Gram-negative bacteria). LPS, lipopolysaccharide.

the activated proteins produced by the series of proteolytic cleavages that comprise the cascade. Regardless of how the complement pathway is activated, the same key activated components are produced: **C3a, C3b, C5a**, and **C5b** (Figure 3-11).

C3a and C5a are proinflammatory molecules that stimulate granulocytes, such as basophils and mast cells, to release the vasoactive substances in their granules, thereby increasing the permeability of blood vessels and facilitating the movement of phagocytes from blood vessels into tissue (Figure 3-2). C5a also acts as a potent chemokine, signaling phagocytes to leave the bloodstream and guiding them to the infection site. Once PMNs or monocytes have left the bloodstream, they move along a gradient of C5a to find the locus of infection.

At the site of infection, C3b binds to the surface of the invading bacterium, enhancing the ability of phagocytes to ingest (engulf) the bacterium. This process of marking phagocyte targets is called **opsonization**, and any molecule that does this marking is referred to as an **opsonin**. As will be seen in the next chapter, antibodies can also perform this function. Without opsonization, phagocytes have difficulty

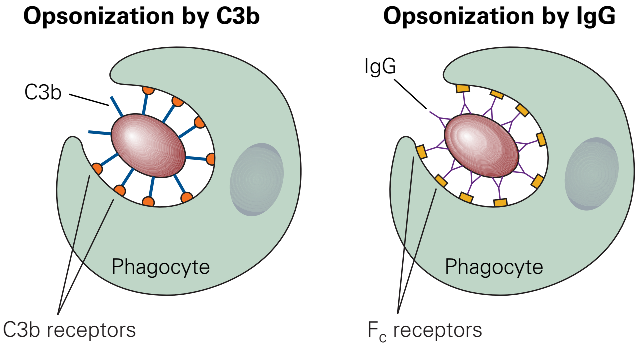


Figure 3-12. Opsonization of a bacterium by activated complement component C3b and antibodies. Combined opsonization by both C3b and antibodies considerably enhances the uptake of the bacterium by phagocytes. IgG, immunoglobulin G.

ingesting a bacterium unless it is trapped in a small space. Because bacteria do not stick well to the phagocyte surface, the action of pseudopod encirclement can actually propel the bacterium away from the phagocytes—analogous to the way a fish can slip from your hands as you try to grab it. Phagocytes have **C3b receptors** on their surfaces that specifically bind C3b (Figure 3-12). Thus, by binding to the bacterial surface, complement component C3b gives the phagocyte something to grab on to as it tries to engulf the bacteria. Antibodies bound to bacterial surfaces can also act as opsonins, because a conserved portion of the heavy chain of the antibody, the Fc region, is recognized by **Fc receptors** on the phagocyte. The difference between these two processes is that antibodies bind to specific molecules on the surface of a bacterium, whereas C3b binds nonspecifically to glycosyl groups commonly found on bacterial surfaces. The combined effect of C3b and Fc receptors binding to their respective ligands on the bacterial surface works synergistically to enhance phagocytosis.

Activated complement components can also lead to direct killing of bacteria. Activated component C5b binds to the O-antigen of LPS of Gram-negative bacteria and recruits sequentially C6, C7, C8, and multiple C9 proteins to form a MAC in the bacterial membranes (Figure 3-13). Formation of the MAC kills bacteria by creating holes in their membranes. Bacteria that can be lysed by MAC are said to be **serum sensitive**, i.e., the addition of serum-containing complement components to the bacteria will kill the bacteria. Some Gram-negative bacteria have altered LPS O-antigens that do not bind C5b and therefore cannot form a MAC on their surfaces, while others have extra-long LPS O-antigens that can still bind C5b but cannot form a MAC in the bacterial membrane. Gram-negative bacteria that cannot be lysed by MAC are said to be **serum resistant**, i.e., they are resistant to killing by serum complement via MAC formation. Gram-positive bacteria are inherently serum resistant, because their thick cell walls prevent MAC assembly at the cytoplasmic membrane.

Steps in Complement Activation

The steps in complement activation by each of the three pathways are shown in more detail in Figure 3-14. The **classical complement pathway** (called that because it was the first to be discovered) is initiated when the Fc regions of several antigen-bound IgG molecules or one pentameric IgM molecule bind to the surface of a bacterium and are subsequently cross-linked by **C1**, a multi-protein complex of hexameric C1q, two C1r and two C1s zymogens ($C1qr_2s_2$) (Figure 3-14A). C1q binds directly to the heavy chain of an-

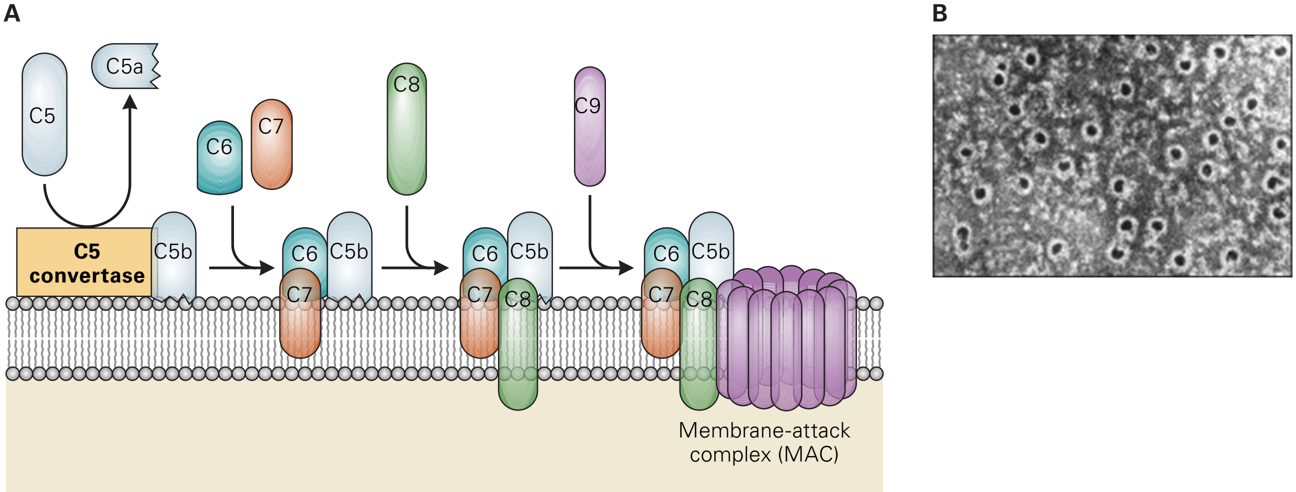


Figure 3-13. Formation of the membrane attack complex (MAC). (A) C5-convertase assembled on the bacterial cell surface cleaves C5 to generate C5a, which is a chemokine for phagocytes, and C5b, which binds to the O-antigen of LPS on Gram-negative bacteria and recruits C6 through C8 to initiate MAC formation and insertion into the membrane. Up to fifteen C9 proteins then oligomerize into the complex. **(B)** MAC pores in the membrane that then kills the bacteria. Reproduced from Janeway C, Travers P, Walport M, Shlomchik M. 2004. *Immunobiology*, 6/e. Garland Science, New York, NY, with permission.

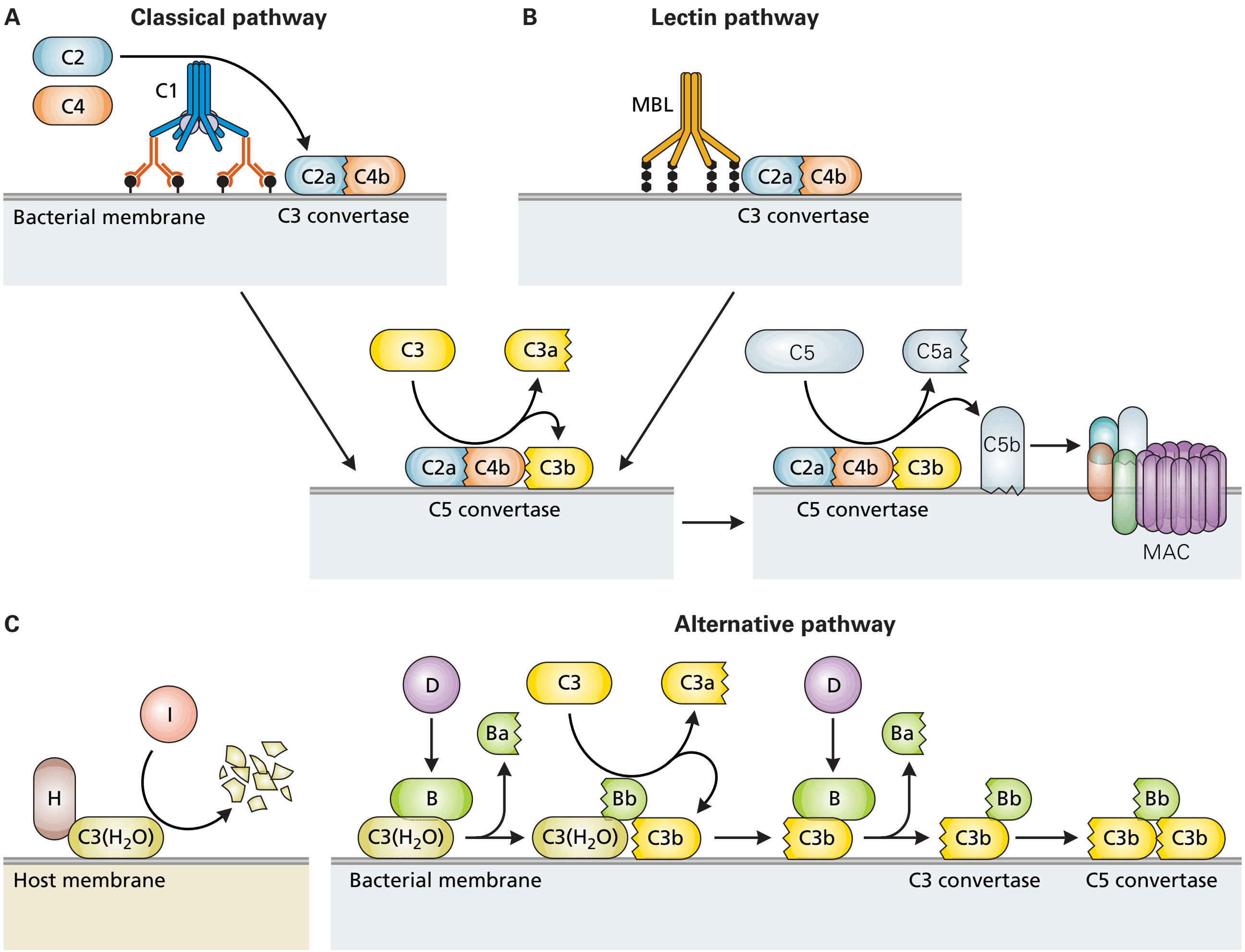

A
Classical pathway
C2
C4
C1
C2a
C4b
Bacterial membrane
C3 convertase
B
Lectin pathway
MBL
C2a
C4b
C3 convertase
C3
C3a
C2a
C4b
C3b
C5 convertase
C5
C5a
C5b
C2a
C4b
C3b
C5 convertase
MAC
C
Alternative pathway
I
H
C3(H2O)
Host membrane
D
B
Ba
C3(H2O)
C3
C3a
Bb
C3(H2O)
C3b
D
B
C3b
Ba
Bb
C3b
Bb
C3b
C3b
Bacterial membrane
C3 convertase
C5 convertase

Figure 3-14. Activation of complement by the classical, lectin, and alternative pathways. (A) Classical pathway: Two IgG molecules or one IgM molecule attached to the surface of a bacterium bind complement component C1, causing an autoproteolytic event that activates it. C1, C4b, and C2a bind to each other and to the bacterium's surface to form C3 convertase. The addition of C3b produces C5 convertase that triggers assembly of the MAC. **(B)** Lectin pathway: The mannose-binding lectins (MBLs) activate the complement pathway similarly to antibodies, except that they interact with C4 and C2 rather than C1. After that point, this pathway is the same as the classical pathway. **(C)** Alternative pathway: C3-H_2O, an activated form of C3 that resembles C3b in conformation, is normally produced at low levels. If it binds a host cell surface through serum factor H, then the H-bound C3-H_2O complex is targeted for destruction by serum protein I. If C3-H_2O binds to the surface of a bacterium, it can form a complex with factor B, which is targeted for cleavage by factor D to generate Ba and Bb. The C3-H_2O complexed with Bb then cleaves more C3 into C3a and C3b. The C3b then covalently binds to the bacterial surface, where it binds factor B, which gets cleaved by factor D to form C3bBb (C3 convertase) on the bacteria surface. Addition of more C3b produces C5 convertase. C5 convertase triggers assembly of the MAC.

tibodies bound to the bacterial surface and activates the two C1r serine proteases, which then in turn cleave and activate the two C1s serine proteases. Activated C1s cleaves C4 into **C4a** and **C4b**. C4b, like C3b, has a reactive thioester group that enables it to covalently attach to the bacterial surface at a site near the C1qr$_2$s$_2$ complex. C1s also cleaves **C2** into **C2a** and **C2b**. C4b binds to C2a to form the **C3 convertase (C2aC4b)**. C3 convertase then cleaves C3 to C3a, which diffuses away from the site, and C3b, which covalently binds to the bacterial surface.

The lectin pathway converges with the classical pathway in that it also stimulates cleavage of C4 and C2, with generation of C4b and C2a that form the C3 convertase complex (Figure 3-14B). The collectin MBL circulates in the blood as a multimeric complex with one or more serine proteases, called **MBL-associated serine protease (MASP) proteins** and **small MASP-associated protein (sMAP)**. MASPs can also bind to ficolins (FCNs). There are three known MASPs: MASP-1, MASP-2, and MASP-3. MASP-1 and MASP-2 resemble C1r and C1s, respectively. When MBL binds mannose groups of glycoproteins found on the surface of many pathogens, the MASP-2 protein cleaves C4 and C2 and initiates formation of the C3 convertase as in the classical pathway. MASP-1 can also cleave C3 directly, whereas MASP-3 appears to associate primarily with FCN but does not appear to activate complement, and its function remains unclear.

Activation via the **alternative pathway** (named because it was discovered after the “classical” pathway) bypasses the need for C1, C2, C4, antibodies, or MBL/MASP, and relies instead on C3b as the initiating component. As C3 circulates in blood and tissue, it is occasionally activated into a water-bound form (C3-H_2O) that can interact with the serum proteins, **factor B** or **factor H**. Tissues of the body are coated with sialic acid residues, that can bind to one end of factor H. In the absence of a bacterial surface, C3-H_2O binds to the other end of factor H to form a complex that produces C3b (Figure 3-14C). This binding of C3b to factor H changes the conformation of C3b and targets it for proteolytic cleavage into iC3b by the serum protease **factor I**. In the absence of a bacterial surface, the iC3b bound to factor H is targeted for further destruction by factor I. However, iC3b can still covalently bind to a bacterial surface via its internal thioester group and as such can serve as an opsonin, even though iC3b can no longer form active C3 convertase.

If C3-H_2O instead binds to serum protein **factor B**, then another serum protein **factor D** cleaves that B to **Ba** and **Bb**. The resulting complex of C3-H_2O bound to Bb cleaves another C3 molecule to produce C3a and C3b, that then covalently binds to the bacterial surface. This membrane-bound C3b binds another factor B, and the complex C3b-B is then targeted for cleavage by factor D to form the **C3 convertase C3bBb**, which in turn continues to cleave more C3 to make more C3a and C3b. C3bBb, which normally has a relatively short half-life of about 90 minutes, is further stabilized by binding of the protein **properdin (P)**, which increases the half-life of the resulting C3bBbP complex by up to ten-fold. Properdin is a soluble glycoprotein released by PMNs, monocytes, and adaptive immune cells in response to the presence of proinflammatory cytokines such as TNF-α.

Generation of large amounts of C3b has two functions. A portion of the C3b binds to the bacterial surface and acts as an opsonin to enhance uptake by phagocytes, while another portion binds to the existing C3 convertase complexes (C3bBb or C2aC4b) to form the C5 convertase complexes (C3bBbC3b or C4bC2aC3b), that then cleave C5 into C5a and C5b. C5a diffuses away from the site and acts as a chemokine to recruit phagocytes, whereas C5b binds to LPS on the surface of Gram-negative bacteria and recruits C6, C7, C8, and C9 to form the MAC. Some bacteria produce a polysaccharide surface coating, called a **capsule**, that preferentially binds serum factor H rather than factor B. As a result, C3b is eliminated as it deposits on the surface, effectively preventing opsonization of the bacterial surface, as well as MAC formation.

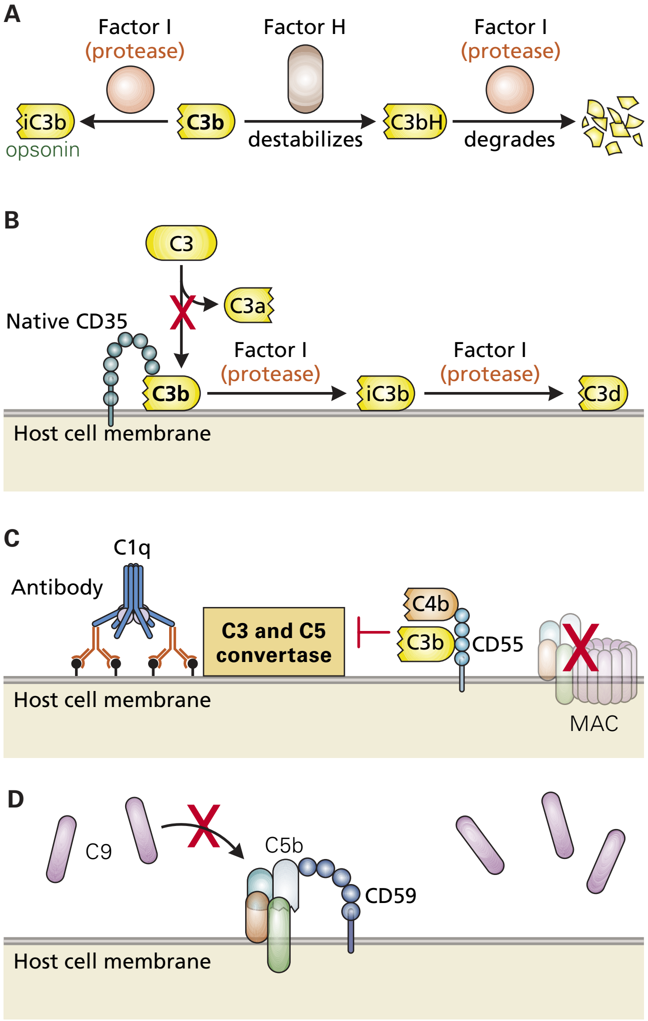


Figure 3-15. Controlling complement. Multiple regulatory pathways help to dampen complement activation, returning the signaling pathways to the resting state. **(A)** Factor H and factor I work together to limit the amount of C3b present in the circulation. Factor H competes with factor B for binding to C3b. Formation of complex C3bH destabilizes C3b, exposing a site for the protease activity of factor I to then completely degrade C3b. C3b can also be clipped to form iC3b, which still acts as an opsonin but no longer stimulates complement. iC3b also is degraded by factor I. **(B)** CD35 (CR1) binds to complement component C3b (as well as C4b) and dissociates the C3 convertase complex, thereby preventing further cleavage of C3 to C3a and C3b. CD35 is also a cofactor for factor I, which clips C3b (as well as C4b) to generate iC3b (and iC4b) and further degradation products. **(C)** CD55 binds to C3b and C4b and causes dissociation of the C3 and C5 convertase complexes, thereby preventing further cleavage of C3 and C5 and limiting the formation of C3a, C3b, C5a, and C5b–C9 (MAC). **(D)** CD59 binds the C5b–C8 complex, which is inserted into the cell membrane, and thereby blocks the binding of C9 to the complex and the polymerization of C9 to form membrane pores.

Controlling Complement Activation

In all three pathways, it is important to keep the accelerated production of C3a, C3b, C5a, and C5b under control so that overstimulation of the inflammatory response does not occur and host cells are not damaged. To this end, several mechanisms protect host cells from complement over-activation (Figure 3-15). Excess C3b molecules that bind to factor H when bound to sialic acid groups on the surface of host cells are proteolytically cleaved to produce **iC3b**. As mentioned previously, while iC3b is an effective opsonin (Figure 3-15A), it can no longer aid in the formation of a C3 convertase or C5 convertase. Likewise, when iC3b is bound to factor H on host cell surfaces, it is subject to further degradation by serum factor I.

Once the pathogen has been cleared from the system and the heightened immune responses are no longer needed, the host cell uses additional regulatory proteins to help inhibit the complement pathways and return them to basal levels of activity. Several proteins protect host cells from complement-mediated damage by rapidly inactivating components of the complement pathways (Figure 3-11). **CD35 (complement receptor 1, CR1)** is a membrane glycoprotein found on phagocytic cells that binds to opsonins on bacterial cells and mediates their phagocytosis (Figure 3-15B). CD35 thus serves as the primary pathway for processing and clearing complement-opsonized immune complexes and inhibits both classical and alternative pathways. **C4b-binding protein (C4BP)**, a glycoprotein found in blood plasma that binds C4b (and, to a lesser extent, C3b), is an inhibitor of the classical and lectin complement pathways. C4BP serves as a cofactor of serum factor I, accelerating the degradation of C3 convertase by factor I-mediated cleavage of C4b and C3b. **CD46 (membrane cofactor protein)** is also a membrane protein receptor that binds factor I as a cofactor to cleave and inactivate C3b and C4b.

Several factors help protect the host cell from being damaged by complement activation by blocking MAC from forming on the host cell surface and thereby protecting the host cell membrane (Figure 3-15C and D). **CD59 (MAC-inhibitory protein, MAC-IP)** is a membrane-associated glycoprotein that blocks C9 polymerization and MAC formation. **CD55 (complement decay-accelerating factor, DAF)** is a membrane-associated glycoprotein found on the surface of many blood cells. CD55 binds to C4b and C3b and interferes with their ability to bind C2b and Bb to prevent formation of the C3 convertases (C4bC2b and C3bBb, respectively) on the host cell surface, thereby protecting the host cell membrane.

Cytokines and Chemokines—Mediators of Immune Responses

Roles of Cytokines and Chemokines in Directing Innate Immune Responses

Cytokines and chemokines play a central role in regulating the cellular activities of both the innate and adaptive defense systems (Table 3-2). These signaling molecules act as messengers by binding to receptors on the cells whose activities they direct. Cytokines are soluble glycoproteins (see Box 3-1) of 8 to 30 kDa produced by a variety of cells, including PMNs, DCs, NK cells, endothelial cells, and cells of the adaptive immune system, as well as other host cells when they are infected. Chemokines are small glycopeptides of 8 to 10 kDa that are produced by the same cells that produce cytokines. Their main function is to attract and activate phagocytes, a function similar to that of complement components C5a and C3a.

Cytokines and chemokines recognize and bind to specific receptors on the surfaces of target immune cells, that set off signal transduction cascades that modify the functions of the immune cells. Just as complement can be activated by bacterial surfaces, cytokine release can be triggered by interaction between cytokine-producing cells and molecules on the surfaces of the invading bacterium. In the case of Gram-negative bacteria, the outer membrane LPS that activates complement is also a molecule that stimulates cytokine production. Although the surface molecules of other types of bacteria that activate complement and stimulate cytokine release have not been studied nearly as well, it appears likely that the same surface molecules on these bacteria that activate complement also stimulate cytokine production.

During an infection, phagocytes and other cells (such as endothelial cells) release a variety of different cytokines. Some appear early in the infection and are responsible for up-regulation of innate defenses. Others appear late in the infection and help to down-regulate the defense response. Among the earliest-appearing cytokines are **granulocyte-macrophage colony stimulating factor** (**GM-CSF**) and **interleukin-3** (**IL-3**). These cytokines stimulate stem cells to produce monocytes and granulocytes (especially PMNs) and trigger their release from the bone marrow into circulation. Other early-appearing cytokines, such as TNF-α, IL-1, **IFN-γ**, and **interleukin-8** (**IL-8**), stimulate the monocytes and granulocytes to leave the bloodstream and migrate to the site of infection. The steps in this process for PMNs are illustrated in Figure 3-2.

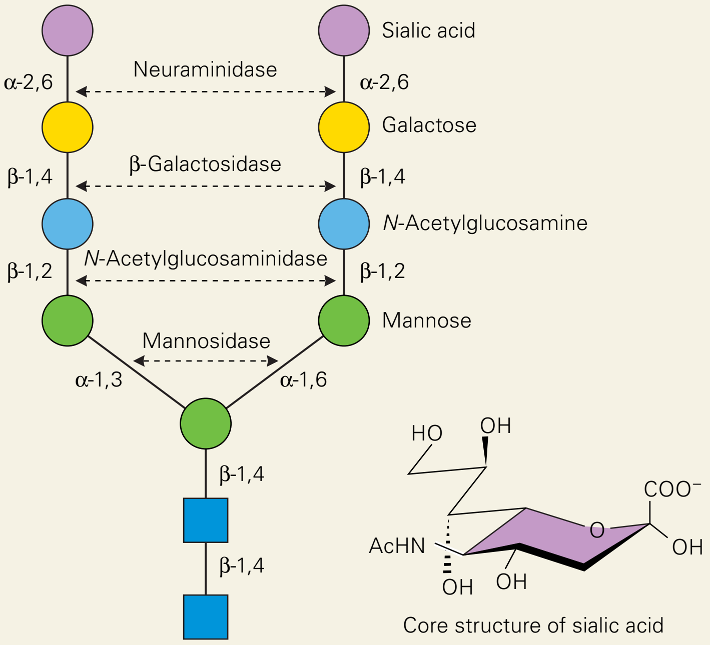
Normally, PMNs move rapidly through the blood vessels, occasionally colliding with one of the vessel walls. TNF-α, IL-1, and IFN-γ stimulate endothelial cells to produce a set of surface proteins called **selectins**. These selectins bind to proteins on the surface of PMNs and other phagocytic cells in the blood, causing them to loosely attach to the blood vessel endothelium. This loose attachment slows the movement of the blood cells, causing them to roll along the endothelial surface. As this occurs, other selectins will appear on the endothelial cells, while IL-8 stimulates the PMNs to produce proteins called **integrins** on their surfaces. The integrins bind another set of cytokine-stimulated proteins on the endothelial cell surface, the **intercellular adhesion molecules** (**ICAMs**), to generate a tighter attachment between PMNs and endothelial cells. This tight association stops the movement of the PMNs and causes them to flatten against the blood vessel wall. The slowing and stopping of the PMNs

Box 3-1.

Glycoproteins

The surfaces of human cells are coated with glycoproteins that, as the name implies, contain proteins covalently linked to oligosaccharides. These glycoproteins play diverse roles in eukaryotic organisms. Shown are the structures of a typical N-glycosylation structure on human glycoproteins, that are capped with the nine-carbon monosaccharide sialic acid, called neuraminic acid. The predominant sialic acid found in mammalian cells is N-acetylneuraminic acid (core structure shown below). Not surprisingly, bacteria produce enzymes on their surfaces that can hydrolyze and release the sugars from host glycoproteins, such as the proteins NanA, BgaA, and StrH from *S. pneumoniae*, and provide food to the bacteria as well as opening up binding sites for bacteria to attach to the host cell surface.

Source:
King SJ, Hippe KR, Weiser JN. 2006. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. *Mol Microbiol* **59:**961–974.



Core structure of sialic acid

is called **margination**. The PMNs then force themselves between endothelial cells, a process that is assisted by a PMN protein called **platelet-endothelial cell adhesion molecule** (**PECAM**). The proinflammatory complement components C3a and C5a assist in the process of transmigration (diapedesis, or extravasation) from the bloodstream into tissue by causing mast cells to release **vasoactive** histamine and heparin, which dilate blood vessels and make them leakier. Dilation of blood vessels is also assisted by the cytokine **platelet-activating factor** (**PAF**). In addition to histamine and heparin, PAF triggers mast cells to produce a number of other vasoactive compounds from the membrane lipid, arachidonic acid, including various leukotrienes and prostaglandins. Once the PMNs have moved out of the blood vessel and into surrounding tissue, a gradient of soluble complement components, chemokines or bacterial peptides, leads them to the site of infection (through chemotaxis).

As PMNs move through tissue, the proinflammatory cytokines TNF-α, IL-1, IL-8, and PAF also activate the oxidative burst response in PMNs so that the PMNs arrive at the infection site with their full killing capacity in place. Monocytes and the macrophages into which they develop similarly activate as they move into an infected area. IFN-γ further stimulates the killing ability of macrophages, producing activated macrophages. Because the signals that control the activities of PMNs, cytokines, and activated complement components are at their highest concentrations near an infected area, PMNs will exit the blood vessel near the location of infection rather than in other areas of the body. Additionally, the fact that activation of the phagocytes occurs only as they are moving into the infected area minimizes the amount of collateral damage to tissues outside the infected area.

The end result of the signaling pathways just described is that a high number of PMNs and other phagocytic cells will leave the blood vessels near the site of infection. However, there are some underlying conditions that will reduce the effectiveness of this signaling system and reduce PMN transmigration, including steroid use, stress, hypoxia, and alcohol abuse. Their inhibitory effect on transmigration may explain why these underlying health conditions are frequently associated with increased susceptibility to infection.

If the phagocytes are successful in eliminating the invading bacterium, other cytokines begin to predominate. In addition to the inhibitory regulators of the complement cascade mentioned earlier, a number of anti-inflammatory cytokines, such as IL-4, IL-10, and **interleukin-13 (IL-13)**, down-regulate production of TNF-α and reduce the killing activities of phagocytes, thereby countering the proinflammatory response and allowing the phagocyte defense system to return to its normal, relatively inactive level.

Inflammation and Collateral Damage

Inflammation, derived from the Latin word *inflammare*, which means "to set on fire," is the immunologic and vascular response of the body to invasion by pathogens. Inflammation is caused by the release of proinflammatory cytokines, including C3a and C5a generated by the complement cascade; **tumor necrosis factor alpha (TNF-α)**, **interleukin-1 (IL-1)**, and **interleukin-6 (IL-6)** released by basophils, mast cells, activated DCs, macrophages, and other phagocytes; and subsequent production of prostaglandins and leukotrienes. Other inflammatory mediators include the vasoactive peptides **kallidin** and **bradykinin**, which dilate blood vessels leading to a drop in blood pressure and increase vascular permeability at the site of infection to allow neutrophil and monocyte transmigration (extravasation).

Although inflammation serves to protect and control infections, it can also cause further tissue damage, which is manifested as the disease symptoms of redness, swelling, heat, and pain. The increased blood flow to the area due to vasodilation results in redness and elevated temperature. The increased vascular permeability causes blood fluids to leak out of the vessels as the phagocytes transmigrate, causing edema (swelling) of the surrounding tissue. The source of the pain is still not clearly understood, but it is likely caused by the combined effects of cytokines, prostaglandins, and coagulation cascade components on nerve endings in the inflamed region. Bradykinin also appears to increase sensitivity to pain. To counter the proinflammatory response, there are a number of **anti-inflammatory cytokines**, including **IL-1 receptor antagonist**, **interleukin-4 (IL-4)**, and **interleukin-10 (IL-10)**, that serve to regulate the immune response by inhibiting the action of the proinflammatory cytokines.

Although phagocytic cells are effective killers of bacteria and are essential for clearing the invading bacteria from an infected area, the body can pay a high price for this service. During active killing of a bacterium, lysosomal enzymes are released into the surrounding area as well as into the phagolysosome. Released lysosomal enzymes damage adjacent tissues and can be the main cause of tissue damage that results from a bacterial infection. Also, PMNs, NK cells, and macrophages kill themselves as a result of their killing activities, and lysosomal granules released by dying PMNs contribute further to tissue destruction. **Pus**, a common sign of infection, is composed mainly of dead PMNs and tissue cells. If pus accumulates in an enclosed tissue area, then an **abscess** can form. Pus is usually whitish or yellowish in color, but sometimes it can have a greenish color due to the abundant presence of myeloperoxidase. Bacteria that cause pus formation are called **suppurative**, **purulent**, or **pyogenic**.

Some of the other roles of the proinflammatory cytokines are listed in Table 3-2 and illustrated in Figure 3-16. These proinflammatory cytokines are responsible for common symptoms of infectious diseases other than those localized at the site of infection: fever, somnolence (drowsiness), malaise, anorexia, chills, decrease in blood iron levels, and weight loss. Cytokines IL-1 and TNF-α interact with the hypothalamus and adrenal gland to produce fever and somnolence, which the patient interprets as a feeling of malaise. Indifference to food, which can also characterize this state, contributes to the anorexia. TNF-α and other cytokines stimulate muscle cells to increase their metabolic rate and catabolize proteins to provide fuel for the mobilization of host defenses. Increased metabolism of muscle cells may be the cause of chills seen in some types of systemic infections. If the infection persists, the combination of anorexia and muscle cell breakdown of protein results in weight loss and visible loss of muscle tissue (wasting syndrome).

The cytokine IL-6 released from macrophages and T cells stimulates the liver to increase production of transferrin (to sequester iron), complement components (to regenerate complement components used up during complement activation), LPS-binding proteins (to continue stimulation of cytokine production as long as bacteria are detected in the body), and two general opsonins—MBL, discussed above in the context of complement, and **C-reactive protein (CRP)**. CRP is a pentameric protein produced in the liver whose expression and release into blood is greatly up-regulated in response to IL-6 during inflammation. CRP is also another PAMP recognition protein in that it binds to LTA of some bacterial species as well as to phosphocholine found on the surface of dead or dying bacterial cells. Phagocytes have receptors for CRP, which allows it to act as an opsonin by enhancing phagocytosis.

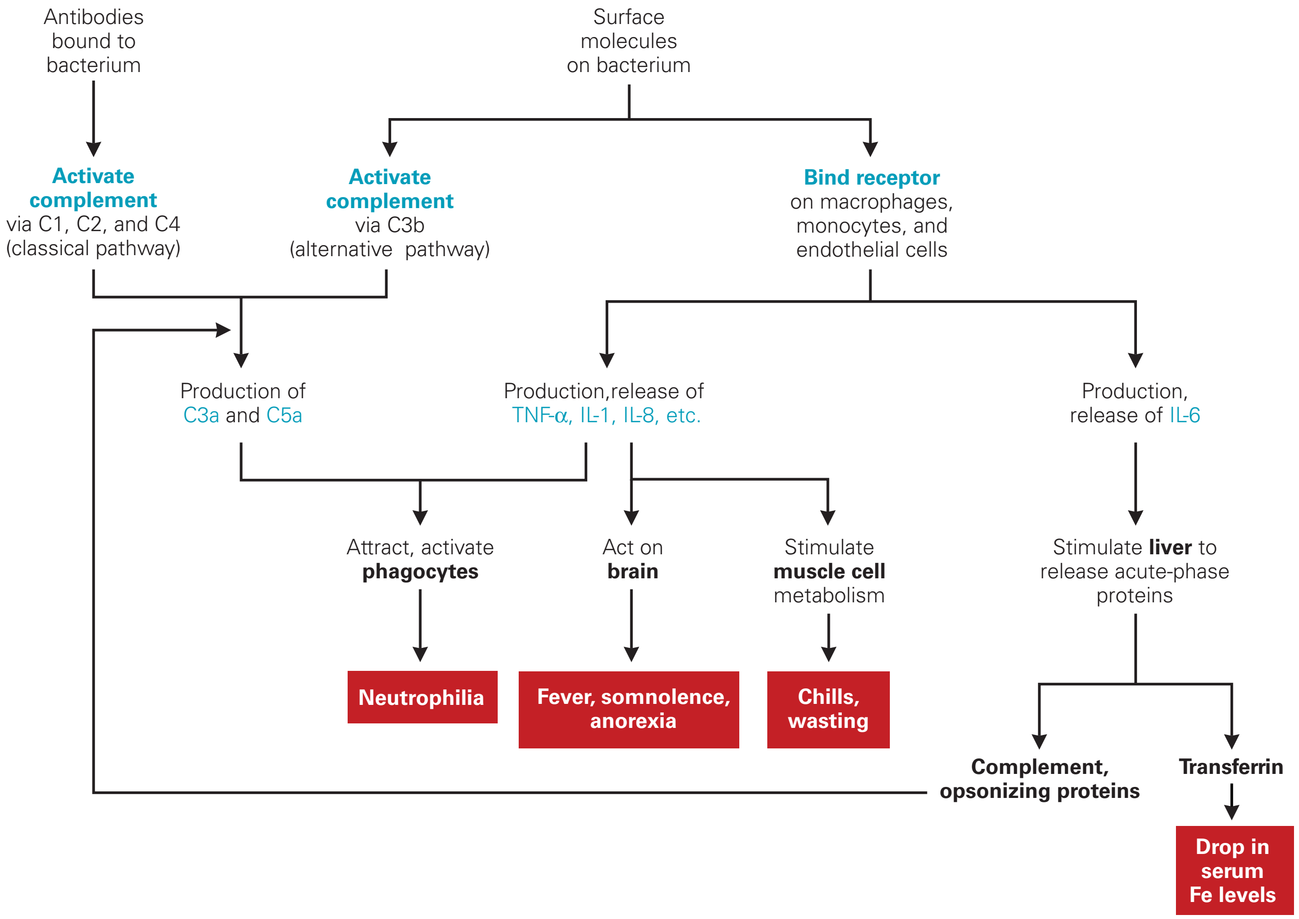

Antibodies bound to bacterium
Surface molecules on bacterium
Activate complement via C1, C2, and C4 (classical pathway)
Activate complement via C3b (alternative pathway)
Bind receptor on macrophages, monocytes, and endothelial cells
Production of C3a and C5a
Production, release of TNF-α, IL-1, IL-8, etc.
Production, release of IL-6
Attract, activate phagocytes
Act on brain
Stimulate muscle cell metabolism
Stimulate liver to release acute-phase proteins
Neutrophilia
Fever, somnolence, anorexia
Chills, wasting
Complement, opsonizing proteins
Transferrin
Drop in serum Fe levels

Figure 3-16. Overview of nonspecific responses to infection and the effects of cytokines on these responses. Proinflammatory cytokines include TNF-α, IL-1, IL-6, and IL-8, as well as the complement components C3a, C4a, C2b, and C5a. C3a, C4a, and C5a act as anaphylatoxins that increase histamine and heparin release from mast cells and basophils, smooth muscle contraction, and vascular permeability. C5a and (to a lesser extent) C3a also act as potent chemokines (enhancing chemotaxis of phagocytes to the infection site).

Septic Shock: The Dark Side of the Innate Defenses

Inappropriately functioning or over-stimulated host defenses can sometimes lead to disastrous consequences such as **septic shock**. Septic shock is a form of shock caused by bacterial infection (Figure 3-17), during which a massive release of cytokines (sometimes called a cytokine storm) leads to a subsequent overwhelming inflammatory response. Other causes of shock include massive crush injuries, burns, radiation poisoning, and exposure to toxins (**toxic shock**). In people with septic shock, vasodilation causes a dramatic drop in vascular resistance and blood pressure (**hypotension**) despite normal-to-high cardiac output. The heart rate and respiratory rate increase as the body tries to compensate for decreasing blood pressure and lack of oxygen. Septic shock is a serious condition because severely reduced levels of blood flow deprive essential organs of oxygen and nutrients (**ischemia**), causing them to malfunction. The consequent failure of multiple organs, such as the kidneys, heart, brain, and lungs, is the usual cause of death in septic shock patients.

Septicemia (bacteria infecting and growing in the bloodstream) is one of the leading causes of septic shock and occurs in 500,000 to 750,000 patients per year in the United States alone. Up to half of the patients with septicemia end up dying in the hospital. Even those who survive may have long-term aftereffects, such as stroke or permanent damage to lungs, kidneys, liver, or other organs. Indeed, statistics show that those who survive an episode of septic shock have a significantly greater risk of dying during the next five-year period than people with the same underlying conditions but no previous episode of shock. Septic shock is not only serious business for the patient, but it is also a seriously expensive business, as hospital administrators and insurance executives are quick to point out, with annual hospital costs of $5 to 10 billion.

The all-inclusive term sepsis has now been defined more precisely in an effort to aid diagnosis of the various stages

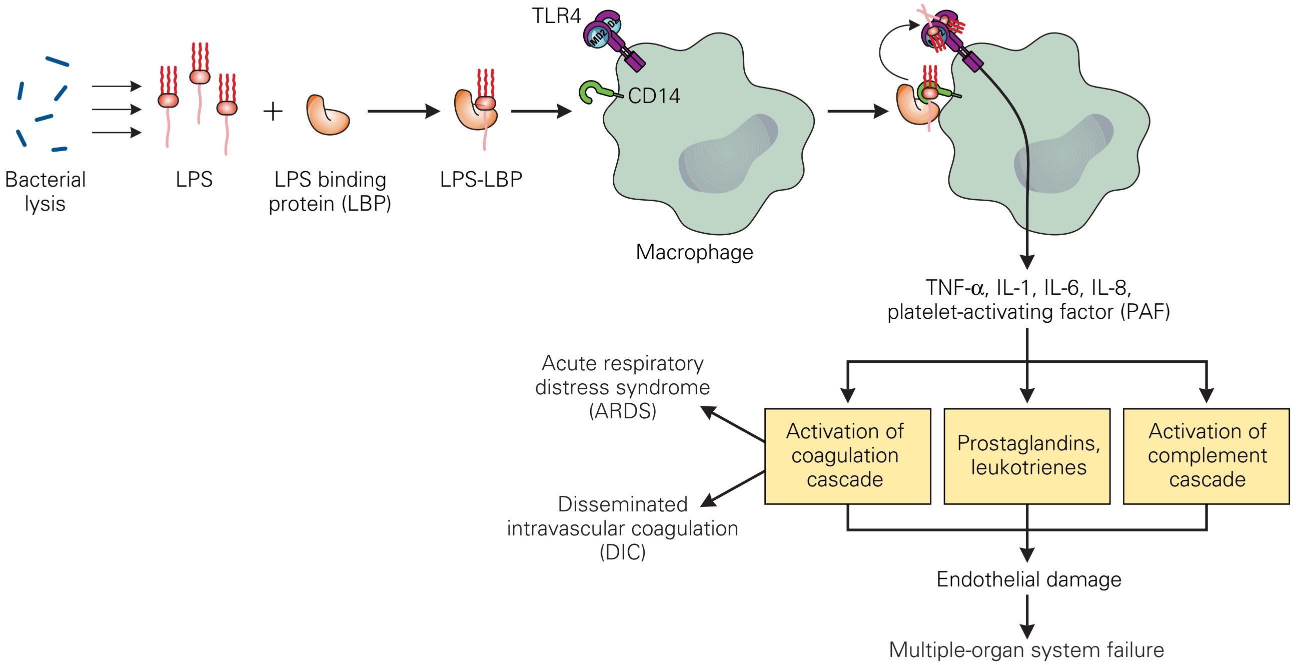

Bacterial lysis
LPS
LPS binding protein (LBP)
LPS-LBP
TLR4
MD2
CD14
Macrophage
TNF-α, IL-1, IL-6, IL-8, platelet-activating factor (PAF)
Acute respiratory distress syndrome (ARDS)
Disseminated intravascular coagulation (DIC)
Activation of coagulation cascade
Prostaglandins, leukotrienes
Activation of complement cascade
Endothelial damage
Multiple-organ system failure

Figure 3-17. Triggering of proinflammatory cytokine release by Gram-negative LPS and its role in septic shock. TNF-α, IL-1, IL-6, and IL-8 are proinflammatory cytokines.

of disease. The first stage of shock, called **systemic inflammatory response syndrome** (**SIRS**), is characterized by a temperature over 38°C or under 36°C, elevated heart rate, elevated respiratory rate, and an abnormally high or low neutrophil count. The second stage, termed **sepsis,** is SIRS with a culture-documented infection (i.e., laboratory results showing the presence of bacteria in the bloodstream). The third stage is **severe sepsis**, characterized by organ dysfunction and very low blood pressure (hypotension). The fourth stage is **septic shock**, which is characterized by low blood pressure despite intravenous fluid administration.

How does a bacterial infection of the bloodstream produce such a serious condition? Steps involved in septic shock are illustrated in more detail in Figure 3-18. From previous sections of this chapter, it should be evident that the body goes to great lengths to confine the inflammatory response to specific areas of the body. Septic shock is an example of what happens when an inflammatory response is triggered throughout the body. Shock occurs when bacteria or their products reach high enough levels in the bloodstream to trigger complement activation, cytokine release, and the coagulation cascade in many parts of the body. High levels of cytokines, especially TNF-α, IL-1, IL-6, IL-8, and IFN-γ, cause increased levels of PMNs in the blood and encourage these PMNs to leave the blood vessels throughout the body. This leads to massive leakage of fluids into surrounding tissue. PMNs and macrophages activated by IFN-γ also damage blood vessels directly, resulting in loss of fluid from these vessels.

Activation of complement throughout the body further increases the transmigration of phagocytes. The vasodilating action of C3a, C5a, leukotrienes, and prostaglandins contributes to leakage of fluids from blood vessels and further reduces the ability of blood vessels to maintain blood pressure. Some cytokines cause inappropriate constriction and relaxation of blood vessels, an activity that undermines the ability of the circulatory system to maintain normal blood flow and blood pressure.

Another sign that is frequently associated with septic shock is **disseminated intravascular coagulation (DIC)**, which can be seen as blackish or reddish skin lesions (**petechiae**). DIC results from widespread activation of **blood clotting** (**coagulation system**) in blood capillaries throughout the body. A mediator of DIC is the release of **tissue factor (TF)**, a glycoprotein found on the surface of many cells, such as endothelial cells, monocytes, and macrophages. TF is released into the circulation in response to proinflammatory cytokines (IL-1 and TNF-α), as well as LPS-stimulated TLR4 signaling during sepsis from Gram-negative bacteria. As clots form, blood flow to organs is impaired and ischemia ensues, leading to the tissue damage seen in DIC. On the other hand, as clotting factors are consumed during the formation of multiple clots, bleeding results in other areas where vasodilation has occurred. In addition, activation of the clotting cascade releases excess thrombin, a protease that cleaves the zymogen plasminogen to plasmin, which in turn causes **fibrinolysis** (the breakdown of clots) and results in **hemorrhaging** (bleeding), a common symptom of DIC.

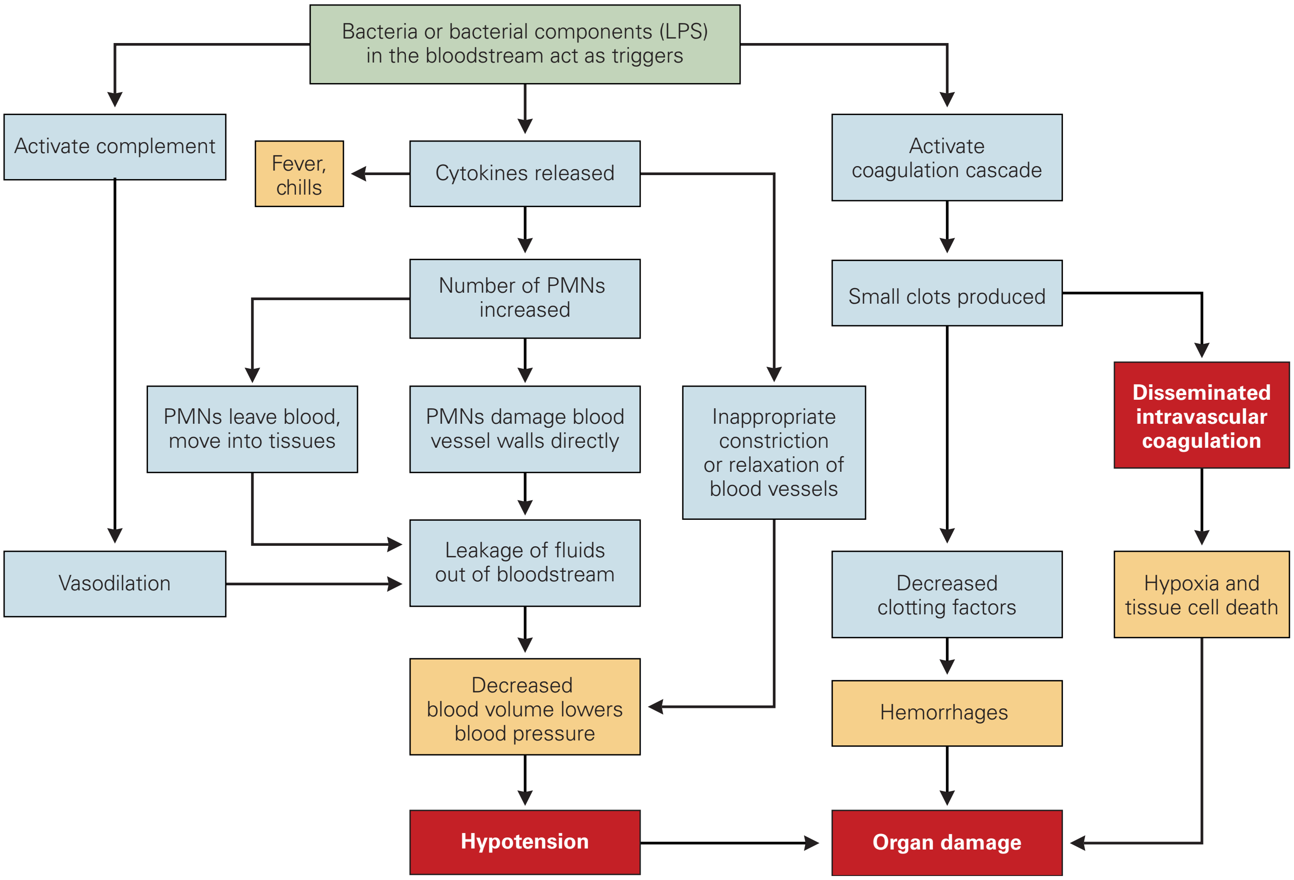

Bacteria or bacterial components (LPS) in the bloodstream act as triggers
Activate complement
Fever, chills
Cytokines released
Activate coagulation cascade
Number of PMNs increased
Small clots produced
PMNs leave blood, move into tissues
PMNs damage blood vessel walls directly
Inappropriate constriction or relaxation of blood vessels
Disseminated intravascular coagulation
Leakage of fluids out of bloodstream
Vasodilation
Decreased clotting factors
Hypoxia and tissue cell death
Decreased blood volume lowers blood pressure
Hemorrhages
Hypotension
Organ damage

Figure 3-18. **Sources of hypotension, disseminated intravascular coagulation (DIC), and internal hemorrhages seen in cases of septic shock.**

Hemorrhages not only contribute to hypotension but also damage vital organs.

Once septic shock enters the phase in which organs start to fail, it is extremely difficult to treat successfully. The death rate for severe septic shock is 30–50%, but DIC worsens the prognosis to 70–80%. Treatment is most likely to be effective if it is started early in the infection; however, diagnosis of septic shock in its early stages is not straightforward, as the early symptoms of shock (fever, hypotension, and tachycardia) are very nonspecific. Also, the transition from the early stages to multiple organ failure can occur with frightening rapidity. Of the hundreds of thousands of cases of septic shock that occur in the United States each year, many occur in patients hospitalized with some underlying condition other than infectious disease, which complicates the treatment options for patients.

Accordingly, a massive effort has been made to develop new techniques for treating septic shock patients more successfully, especially in cases where the disease has reached the point at which treatment with antibacterial agents is no longer sufficient to avert disaster. Early efforts to combat septic shock have centered on the administration of glucocorticoids, which down-regulate cytokine production. Physicians have long believed that administration of steroids or ibuprofen would help shock victims because these compounds dampen various aspects of the inflammatory response, but there is now general agreement that glucocorticoid treatment is not effective in treating most types of shock. Indeed, clinical trials have now shown no significant effects from either of these therapies.

More recently, attention has focused on the proinflammatory cytokines, such as TNF-α, that seem to play a central role in the pathology of shock. Antibodies or other compounds that bind and inactivate cytokines have been tested for efficacy in clinical trials. Although the outcome of early clinical trials has been disappointing, newer anti-cytokine agents now being tested appear to be more promising. Nonetheless, it is clear that this type of therapy will never be as effective as catching septic shock in its very early stages.

One impediment to early diagnosis of shock has already been mentioned: the nonspecific nature of the signs and symptoms of shock. Another impediment to early diagnosis is that so many different types of bacteria can cause septic shock. If diagnosed early enough, intensive antibiotic treatment can be effective in halting the shock process. However, until recently, no definitive microbial diagnosis could be made in about a third of patients with clinical signs of sepsis. Additionally, although bacteria are the microorganisms most frequently implicated in septic shock (approximately 80% of cases), many different species of Gram-positive and Gram-negative bacteria can cause shock. Because no single antibiotic is effective against all of these bacterial pathogens, it is important to determine the species of bacterium causing the infection. This is further complicated by the varied natures and mechanisms by which antibiotics work to perform bacterial killing, As we will further explore in chapter 15, antibiotics that result in lysis of the bacterium might cause further harm by causing release of membrane components such as the endotoxin LPS.

Fortunately, some new methods for rapid bacterial identification have been approved by the FDA and others are in late-stage development. These methods rely on highly sensitive technologies, such as mass spectroscopy and PCR-coupled high-throughput sequencing, which enable determination of protein or DNA content from samples taken directly from blood or cultured from blood for only a few hours. The protein or DNA profiles obtained are then quickly compared to library databases of hundreds of standard profiles from known bacterial pathogens. These new methods make identification of pathogens possible in less than a day, and sometimes in just a few hours, instead of the several days required by standard microbiological testing. Other research efforts are focused on determining biomarkers indicative of different causes of sepsis to guide appropriate antibiotic administration and shock treatments. We will discuss more of these methods in chapter 9.

Other Innate Defenses of the Body—Nutritional Immunity

Another innate defense that is present in blood and helps to hamper spread of the invading microbes is a group of proteins that sequester metals away from bacteria that enter the body. Transition metals, such as iron, manganese, and zinc, are essential to the survival of invading bacteria. By restricting metal availability through a group of metal-binding proteins as part of the innate defenses, the body is able to prevent the growth of invading microbes. This limitation of essential nutrient transition metals, termed **nutritional immunity**, is gaining recognition as a potent innate defense against pathogens.

We have already discussed the role of **lactoferrin** as a secreted iron-binding protein of surface defenses (milk, tears, saliva, and nasal secretions) that hampers microbial growth and colonization outside the body. Like lactoferrin, **transferrin** sequesters iron and prevents iron acquisition by microbes that enter the blood. Transferrin is a glycoprotein produced by the liver that circulates through blood and lymph and binds extremely tightly to the ferric form of iron (Fe^{3+}), controlling the level of free iron available. Transferrin bound with iron is recognized by a cell surface receptor that transports the Fe^{3+}-transferrin complex into recycling endosomes, where the iron is sequestered and removed from the transferrin, and the transferrin is then recycled to the surface, where it can scavenge more iron. In this way, the levels of iron in blood drop to an even lower level than normal, which severely limits the growth of most bacteria. Transferrin and lactoferrin, along with their receptors, are also thought to mediate cellular uptake of other divalent metal ions, such as manganese, zinc, and copper, albeit to a lesser extent.

Manganese, like iron, is required for many essential enzymes in bacteria. Given its importance for bacterial growth, the host has also devised strategies to restrict bacterial invaders from acquiring manganese. In phagosomes, the divalent-metal ion transporter **NRAMP1** is an integral membrane protein that depletes manganese, iron, and other essential metals from the phagosomal compartment to effectively starve the engulfed bacteria. Genetic mutations in the gene encoding NRAMP1 are associated with susceptibility to tuberculosis, leprosy, inflammatory bowel disease, and autoimmune diseases.

Calprotectin is an abundant cytoplasmic protein that is constitutively expressed in neutrophils, but it can also be expressed in other cell types during infection. Extracellular concentrations of calprotectin can exceed 1 mg/ml at sites of infection. Calprotectin acts as a dimer of two proteins (S100A8 and S100A9) that bind and sequester the essential nutrients manganese and zinc with high affinity. The sequestration of these metal ions by the host inhibits pathogen growth and can render the invading bacteria more sensitive to the oxidative burst produced by phagocytic cells. Calcium binding enhances the affinity of calprotectin for manganese and zinc. This suggests that calcium functions as a molecular switch, keeping the protein in a low affinity state within the host cell cytoplasm, where calcium concentrations are very low, but allowing calprotectin to convert into a high affinity state in the calcium-replete extracellular medium, where it can chelate and sequester manganese and zinc once it is mobilized and released from the cells.

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Questions

1. What are two types of bacterial killing mediated by complement?
2. In the activation of complement by the alternative pathway, the stabilization of one particular component is essential. What is this component? How is it stabilized?
3. Define opsonization and two components of the immune system that can act as opsonins. Are they acquired or are they are innate?
4. How do the functions of various activated complement components resemble the functions of cytokines and chemokines?
5. Why does the body have such a complex system (complement plus cytokines) for directing the activities of the phagocytes? What is this system trying to achieve?
6. Why are there two pathways for activating complement?
7. What is the point of using proteolytic cleavage to activate complement? Why not just have the molecules made in their active form?
8. Why does blood pressure fall during septic shock?
9. Why are antibiotics (chemicals that kill bacteria) ineffective after a certain point in the course of septic shock, even though the causative bacterium is susceptible to them? Make an educated guess as to why so many anticytokine therapeutic agents fail.
10. What kinds of evasive action could a bacterium take to prevent it from being killed by a phagocyte? (Hint: Consider the steps in the killing process.)
11. Why do neutrophils circulate in blood? Why not have them migrate permanently into tissue, as macrophages do?
12. Inflammation near the skin is characterized by redness and swelling. What causes these symptoms?

13. What is the primary function of Toll-like receptors and NOD proteins? Where are they located? How do they stimulate immune responses?

14. What might happen to individuals with genetic defects in the following molecules?
    a. C3 b. C5 c. Factor B d. MBL e. TLR4
    f. transferrin

15. What possible advantages would there be for the intracellular Gram-positive pathogen *Listeria monocytogenes* to grow within the cytosol of a phagocytic cell? What possible advantages would there be for *Salmonella* Typhimurium to reside within its own phagosomes?

16. Name three different specific immune mechanisms that enhance a phagocyte's ability to clear a pathogen from the body. How do they each work to help phagocytic clearing of the pathogen?

17. What are the two primary functions of the lymphatic system?

18. NK cells play important functions in the innate immune response. Provide an explanation for each of the following statements:
    a. NK cells kill infected host cells nonspecifically, but not randomly.
    b. NK cells can recognize infected cells directly and indirectly.
    c. NK cells are similar to, but differ from, CTLs.

Comic Relief

19. How would you attract the attention of a good-looking phagocyte?

20. How would you describe a macrophage that is wearing sunglasses and a camera?

Answers to Comic Relief:

19. Try complements.

20. Phago-sightseer.

4

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CHAPTER 4

The Adaptive Defenses

ANTIBODIES AND CYTOTOXIC T CELLS

If ordinary police are confronted with a dangerous situation that they are unable to resolve, they call in the SWAT (special weapons and tactics) team, specialists such as snipers and hostage negotiators, who are trained to target and deal with a specific crisis. When the innate immune system is faced with a prolonged bacterial attack that it cannot handle, the human body uses a similar strategy. It brings in the specialists: antibody-producing B cells and cytotoxic T cells, also called cytotoxic T lymphocytes (CTLs), that are designed to target and kill a specific invading microbe.

The Specialists: Adapting to a Particular Pathogen Challenge

The innate defenses of the body are normally an effective protective force that eliminates invading microbes. Unfortunately, some microbes have developed strategies for evading these innate defenses and surviving in the host. For example, some microbes are able to resist phagocytosis by neutrophils, while others are even able to survive and multiply within phagocytic cells once ingested. To cope with these microbes, the body has evolved a second defense system, the **adaptive** or **acquired immune response**, which enables the host to recognize specific pathogens and mount a targeted response against them. In this adaptive immune response, **dendritic cells (DCs)** internalize the foreign substances that are then processed into smaller pieces and presented on their cell surface to **T helper (Th) cells**. The Th cells in turn stimulate **B cells** to produce antibodies that specifically recognize the foreign substance, targeting it for opsonization by phagocytes. The Th cells also stimulate **cytotoxic T lymphocytes (CTLs)** that recognize and kill infected host cells.

Unlike the innate immune system, which mounts a generalized response against foreign invaders, the adaptive immune system involves specialized cells of the body that change or adapt to target individual invaders in a highly specific way. As such, the adaptive response takes time to adjust and fully develop its specific response, taking up to a week to appear on the scene in full force. However, upon subsequent encounters with the same invader, the specific defenses appear much more rapidly, needing only a day or two to respond. In terms of disease manifestation, this usually means that during the first encounter with a new pathogen the body may have to struggle to overcome the infection and may exhibit symptoms of disease. But, upon future exposure to the same pathogen, the body is prepared to rapidly eliminate the threat and can combat the infection much faster than it did the first time around. Vaccination, which will be covered in greater detail in chapter 17, is a strategy for priming the body with an adaptive immune response against a particular pathogen so as to launch this more rapid response without actually having to endure the first, more unpleasant, encounter.

Understanding how the components of the adaptive immune system are induced and how they protect the body from infection is important for understanding how vaccines work, how they are designed, and why different vaccines are administered in different ways. Although vaccines have been important in preventing disease, the yield of successful vaccines has been disappointingly small. This is due to the fact that scientists are only now beginning to understand certain important nuances of the adaptive immune response, such as **autoimmunity**, a condition in which the adaptive immune system begins producing an unwanted immune response directed against the host's own body. In this way, just as the innate immune system has its dark side (septic shock) that we discussed in the previous chapter, the adaptive immune system also has its own dark side (**autoimmune reactions**). Obviously, it is important that vaccines do not evoke this dark side. New insights into how the adaptive immune response develops, together with new insights into how to better deliver vaccines, are beginning to break down some of the barriers that have prevented development of vaccines and other treatment strategies against diseases such as AIDS, gonorrhea, chlamydial disease, tuberculosis, and malaria, which are serious causes of morbidity and mortality throughout the world.

In this chapter, we start with a description of two of the main products of an adaptive immune response, antibodies and cytotoxic T cells. We will then delve into the complex series of steps that produce these defenses. Finally, we will discuss how the body is able to "remember" past encounters with particular pathogens so as to more rapidly mount an effective immune response upon subsequent attacks by those pathogens.

B Cells: Producers of Antibodies

The Humoral (Antibody) Immune Response

Antibodies are **immunoglobulin (Ig)** protein complexes produced by mature **B lymphocytes (B cells)**. **Humoral immunity** refers to the production of antibodies by mature B cells, as well as complement, antimicrobial peptides, and other immune components found in body fluids (archaically called the "humors"), but here we will confine the term to mean primarily the antibody response. Humoral immunity involves activation of naïve B cells through one of two pathways. The first pathway is a T-cell-dependent process, which is followed by clonal expansion and proliferation of the activated mature B cells and their terminal differentiation into **plasma cells** (mature, nonproliferating B cells that act as professional antibody-producing factories). Alternatively, T-cell-independent activation of B cells can occur through direct binding of highly repetitive structures on their surfaces, which leads to proliferation, maturation, and production of antibodies. Antibodies generated through this T-cell-independent pathway tend to have lower affinity for their targets than those generated through the T-cell-dependent activation of B cells.

Antibodies are found in blood (serum) and tissue fluids, as well as many bodily secretions (e.g., saliva, mucosal fluids, vaginal fluids, and breast milk). Antibodies carry out a number of the adaptive immune system's critical tasks, including: binding and neutralizing the activities of foreign substances, such as toxins, bacteria, and other pathogens; coating foreign substances for enhanced opsonization and clearance from the body; and targeting infected host cells for the killing action of cytotoxic T cells (CTLs) and natural killer (NK) cells. To understand how different antibodies perform such diverse tasks, it is first necessary to understand how each type of antibody is put together and how they work.

Characteristics of Antibodies and Their Diverse Roles in Preventing Infection

The basic structures of the five types of antibodies (IgG, IgM, IgD, IgE, and IgA/sIgA) are shown in Figure 4-1. The antibody monomer consists of a complex of two heavy chains (H-chains) and two light chains (L-chains). The words "heavy" and "light" refer to the size of the peptide chain, with the H-chain being the larger of the two. The H-chains and L-chains are held together by a combination of disulfide bonds and noncovalent interactions. The H-chains define the type of antibody, with the H-chains for IgE and IgM being longer than those for IgA, IgD, and IgG.

Antibodies have two important regions: an N-terminal **antigen-binding region (Fab)** that harbors the end of the antibody that binds to a substance considered foreign by the body and a C-terminal **constant region (Fc)** that confers host specificity and interacts with host cells. In the most common antibody, **IgG,** the Fc region interacts with complement component C1q via a glycosylated region or with phagocytic cells via an Fc receptor-binding region. The Fab region contains a constant region and a **variable region (Fv)** that binds to a specific antigen. An **antigen** is defined as any material the body recognizes as foreign (nonself) that binds to an antibody. An antigen that binds to an antibody molecule can be an infectious microbe or some protein, nucleic acid, or carbohydrate component of the microbe. Additional examples of types of antigens are proteins, macromolecules, or organs from noncompatible human donors or other animals, as well as molecules from pets, plants, or insects, including dander, pollens, and toxins.

The humoral adaptive immune system's ability to recognize a wide range of antigens and subsequently adapt to new invading pathogens relies on the ability of the B cell population to produce a vast array of antibodies with enormous diversity. This vast diversity of antigen specificity is possible because the **immunoglobulin (Ig)** genes undergo many gene recombination, rearrangement, insertion, deletion, and splicing events from separate, different gene segments encoding different regions of the antibody molecule during B

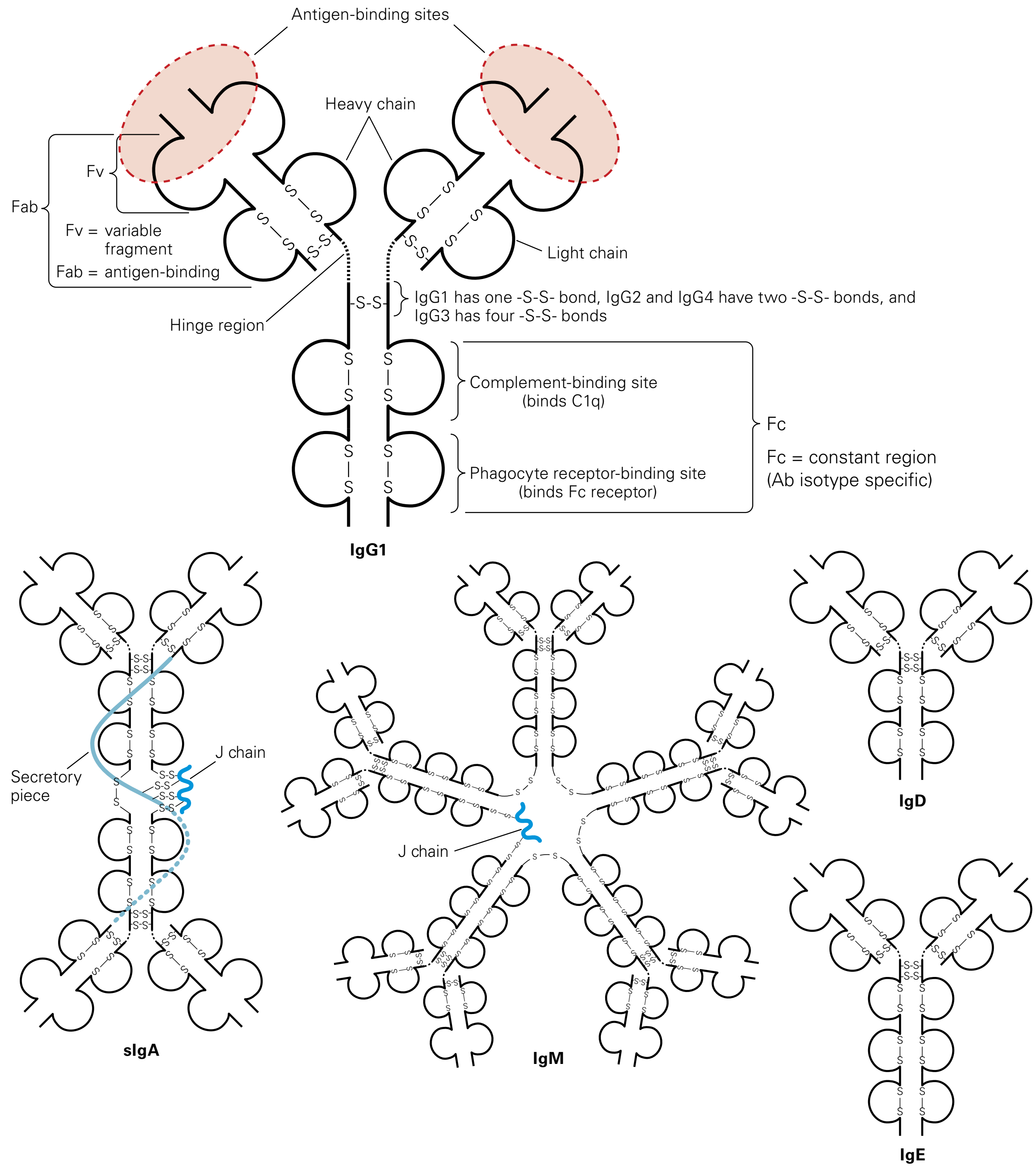
Antigen-binding sites
Heavy chain
Fv
Fab
Fv = variable fragment
Fab = antigen-binding
Light chain
Hinge region
-S-S-
IgG1 has one -S-S- bond, IgG2 and IgG4 have two -S-S- bonds, and IgG3 has four -S-S- bonds
Complement-binding site (binds C1q)
Fc
Fc = constant region (Ab isotype specific)
Phagocyte receptor-binding site (binds Fc receptor)
IgG1
Secretory piece
J chain
sIgA
J chain
IgM
IgD
IgE

Figure 4-1. Structures of IgG, sIgA, IgM, IgD, and IgE antibodies. Each antibody monomer is a complex comprised of four peptide chains: two identical, larger heavy chains (H-chains) and two identical, smaller light chains (L-chains) that are covalently attached through disulfide bonds. The type of H-chain defines the antibody class, with the H-chains for IgE and IgM being longer than those of IgA, IgD, and IgG. IgG is the major class of circulating antibodies. Each monomer recognizes the target epitope via two antigen-binding sites that are located in the variable regions (Fv) of the Fab portion of the monomer. There are four IgG subtypes (IgG1, IgG2, IgG3, and IgG4). The Fc region of the molecule is responsible for complement activation through binding C1q and for enhanced opsonization through binding to phagocyte Fc receptors. IgA has two subtypes: IgA1, which is found mostly in serum, and IgA2, which forms a dimer of two IgA monomers linked via a polypeptide joining chain (J-chain) and is secreted at mucosal surfaces. The IgA dimer acquires a secretory piece during transport through mucosal epithelial cells and is released as secretory IgA (sIgA) into the lumen, where it binds to mucin. IgD is a monomer that is expressed on the surface of mature B cells or is secreted. IgM is a multimer (mostly pentamer) of IgM molecules linked via disulfide bonds and a J-chain. IgM is highly agglutinating (binds and clumps antigens) and strongly activates complement (thousandfold better than IgG). IgE binds to IgE receptors on mast cells and basophils that, upon binding of antigen, trigger release of histamine and inflammatory cytokines.

cell development. A detailed description of the mechanism that generates the large, highly diverse population of B cells, each producing a different specific antibody that can bind to the different kinds of antigens, is beyond the scope of this book. But briefly, an amazing DNA recombination process, called **V(D)J recombination**, randomly shuffles a wide repertoire of variable (V), diversity (D), and joining (J) gene segments that form the N-terminal Fab regions of H-chain proteins (or just V and J gene segments in the case of L-chain proteins) and fuses them to gene segments corresponding to the constant regions in the Ig genes during the development of B cells. The exact pattern of rearrangements occurs independently in each B cell. RNA transcripts of the resulting mosaic Ig genes are further processed to give expression of the specific antibody produced by each B cell in the population. Normally during this developmental process, any B cells that produce Ig molecules that recognize self-antigens are eliminated. All of the antibodies produced by any given B cell have identical antigen-binding sites. But, during clonal expansion, when an antigen and Th cells stimulate a particular subset of B cells, a fairly high rate of spontaneous mutations, termed **somatic hypermutation**, is introduced, which serves to increase the diversity of the antibody pool even more and leads to proliferation of B cells that produce antibodies with high affinity to their cognate antigens.

An antibody monomer has two **antigen-binding sites**, each of which recognizes and binds to the same specific segment of an antigen. The antigen-binding sites are grooves in the antibody Fv ends that only bind tightly to a molecule having one particular structure, called an **epitope** or **antigenic determinant**. An epitope on a protein antigen can vary in size from 4 to 16 amino acids, although most are 5 to 8 amino acids in length. Complex antigens such as microbes contain many possible epitopes, each binding to a different antibody. Epitopes can be **continuous** or **discontinuous**, based on whether the antibody recognizes the primary (linear peptide) or tertiary (topographical) structures of the protein, respectively (Figure 4-2).

An **immunogen** is an antigen that elicits an immune response, but it is important to note that not all antigens are immunogens. In practice, only a subset of the epitopes on an antigen dominates the specific response to that antigen. Why some epitopes are highly **immunogenic** (i.e., elicit a robust antibody or T cell response) while others are only weakly immunogenic is still not well understood. Immunogenicity is often based on the size and complexity of the antigen molecule and is reflected in the antigenicity of different types of macromolecules. In general, proteins are better immunogens than carbohydrates, which are in turn better immunogens than nucleic acids and lipids.

Serum Antibodies

IgG. IgG, produced as a monomer, is the most prevalent type of antibody in blood and extravascular fluid spaces (approximately 80% of circulating antibodies are IgG). IgG is the only antibody type that can cross the placenta (via transcytosis bound to the neonatal Fc receptor, FcRn) and is responsible for protecting an infant during the first six months of life until the infant's adaptive defenses are developed.

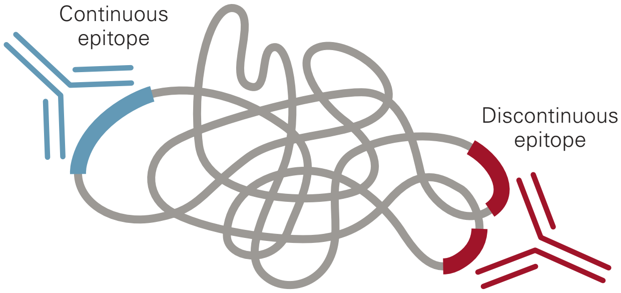

Figure 4-2. Continuous versus discontinuous epitopes. Epitopes of protein antigens can be continuous based on the linear amino acid sequence (primary structure) of the protein or discontinuous based on the tertiary conformational structure of the protein.

There are four different subtypes of IgG antibodies in humans (Table 4-1), named in the order of their abundance in serum: IgG1 (66%), IgG2 (23%), IgG3 (7%), and IgG4 (4%). These subtypes differ not only in their function, but also in their amino acid sequences, glycosylation (post-translational decoration of the IgG protein by sugars), length and flexibility of the hinge region, extent of disulfide bond cross-linking of their H-chains (primarily in the Fc portion), and the specificity of Fc receptor interactions. [Warning: The nomenclature used to describe human IgG is not the same as that used to describe murine IgG. So, IgG1 of mice does not necessarily have the same features as IgG1 of humans. We mention this issue because it explains why different papers on the development of the immune response seem to contradict each other, but actually do not. For a comparative summary of the different types of Ig molecules of humans and mice, see Box 4-1.]

IgG1 and IgG3, but not IgG2 and IgG4, strongly activate the classical pathway of the complement cascade. The six head domains of the C1q complex bind to the Fc region of IgG monomers bound to the surface of the antigen (see Figure 3-14). This cross-linking activates the C1 complex and initiates the complement cascade. IgG1 and IgG3 are also called opsonizing antibodies because these two subtypes are the most effective in opsonizing microbes due to the fact that they have the highest affinity to Fc receptors on phagocytes (see Figure 3-12). IgG2 and IgG4 opsonize poorly, if at all. IgG2 appears to be best at recognizing carbohydrate or polysaccharide-containing antigens. IgG4 appears to play a regulatory role by dampening Fc receptor-mediated inflammatory responses and is produced when persistent exposure to a particular antigen occurs.

Normally, antibody binding to an antigen on the surface of an extracellular pathogen facilitates ingestion of the op-

Table 4-1. **Protective roles of serum antibodies IgG and IgM**

ROLE	IgG1	IgG2	IgG3	IgG4	IgM
Neutralization of toxins	+[a]	+	+	+	+
Neutralization of pathogens (prevents binding of microbe to a target host cell)	+	+	+	+	+
Opsonization; Fc portion binds:					
PMN receptors	+	−	+	−	−
Macrophage receptors	+	−	+	+	+
Complement activation					
Classical pathway	+	−	+	−	+
Alternative pathway	−	−	−	−	+
Cross placenta	+	+	+	+	−
Antibody-dependent cell-mediated cytotoxicity (ADCC)	+	+	+	−	−

[a] +, present; −, absent.

Box 4-1.

Are You a Human or a Mouse?

This archaic challenge, issued to someone showing classic symptoms of soft spine syndrome, in the hopes of getting that person to act decisively and courageously, actually applies to antibodies as well. Although humans and mice are much more closely related than many of us would like to admit, there are subtle but real differences between some antibody classes shared by humans and mice. Accordingly, there is also a somewhat different nomenclature used to designate these antibody classes. These different designations can be confusing, especially in the case of the IgG subtypes. A guide to the different antibody designations in humans and mice is provided below:

HUMAN	MOUSE
IgG1	IgG2a
IgG2	IgG2b
IgG3	–
IgG4	IgG1
IgM1	IgM
IgM2	–
IgE	IgE

Why such differences occur between closely related species remains a fascinating but unanswered question.

sonized antigen by phagocytic cells or triggers complement-dependent killing at the cell surface (see chapter 3). However, in the case of intracellular infections of tissues, IgG (mostly IgG1 and IgG3, but to some extent also IgG2, as well as IgA and IgE) can also mediate a different response, called **antibody-dependent cell-mediated cytotoxicity (ADCC)**. In ADCC, antigens presented on the surfaces of infected cells bind to the Fab regions of specific IgG molecules. The exposed Fc portions of the IgG molecules are then free to bind to effector cells (PMNs, macrophages, or NK cells) that contain Fc receptors on their surfaces. In the case of NK cells, this linkage triggers a cytotoxic bombardment of the infected cell, as described in chapter 3. In the case of macrophages and PMNs a similar killing response can also be elicited, instead of phagocytosis. Thus, by linking together players of the innate (NK cells, PMNs, and macrophages) and adaptive (antibodies) immune responses, ADCC serves as an important defense against intracellular pathogens (more on this later).

IgM. IgM consists of a complex of five or more monomers that are connected via disulfide bonds to each other and to a peptide called the **J-chain** (Figure 4-1). IgM is found mostly in serum, where it accounts for 5 to 10% of the total serum Ig, but it is also secreted at mucosal surfaces and in breast milk. IgM is the most effective activator of complement. Because of its polymeric form, a single molecule of IgM has five Fc regions that can be complexed, which is sufficient to bind C1q and strongly activate complement via the classical pathway or to bind and cluster multiple Fc receptors and mediate enhanced opsonization.

IgM is the first antibody type expressed in the fetus (at 20 weeks' gestation). During B cell differentiation, IgM is the only antibody expressed by naïve B cells. Once B cells mature and proliferate, they start to produce IgG (or IgA) and IgM antibody expression wanes. IgM predominates in the initial (primary) antibody response against a pathogenic microbe, whereas IgG (or IgA at mucosal sites) predominates in response to sustained or subsequent infections (secondary antibody response) by the same microbe (Figure 4-3). Because IgG can circulate in serum for long durations, detectable IgG levels may indicate the presence of a current infection that is well underway or may simply be the residue of a previous infection, whereas IgM levels are detectable during an initial infection but vanish once production of IgG antibodies begins. This aspect of the immune response is actually exploited by diagnostic labs to determine whether a patient is experiencing a first exposure (**acute infection**) to a particular microbe.

IgD. IgD monomer is initially coexpressed with IgM on the surface of mature B cells as they exit the bone marrow and migrate to peripheral lymphoid tissues. IgD appears to function primarily to signal B cell activation.

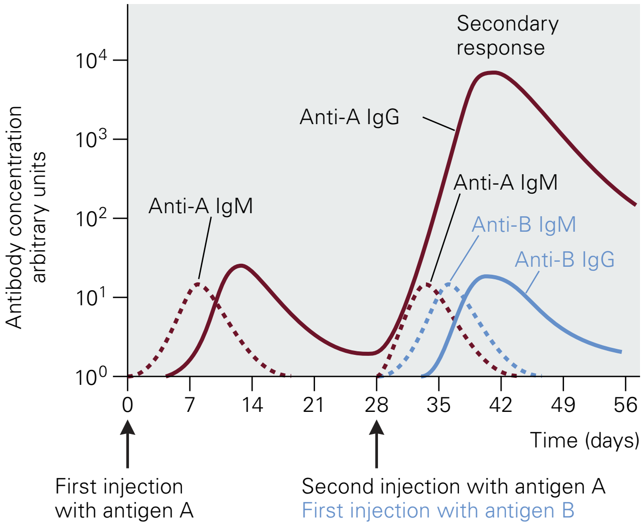

Figure 4-3. The time course of elicitation of antibodies upon initial exposure and subsequent exposure. After exposure to an antigen such as antigen A, IgM is usually the first antibody detected during an acute infection, but levels decrease again after about two weeks. It takes five to seven days for selection and proliferation of B cells producing IgG against antigen A to appear in the blood. After a period of time, the levels of IgG also decrease. Subsequent exposure to antigen A again results in production of IgM at about the same levels as after the first exposure, but the levels of antigen A-specific IgG are now much higher. In contrast, exposure to antigen B along with the second exposure to antigen A does not enhance the antigen B-specific IgG levels.

IgE. A serum antibody with a function different from that of IgG and IgM is **IgE**. IgE is a monomer that is normally found in extremely low concentrations in serum (only 0.05% of total Ig concentration), usually as an Fc receptor-bound complex on the surface of **mast cells** and **basophils**. If two IgE molecules bound to mast cells are complexed by a **polyvalent antigen** (i.e., an antigen with multiple antibody-binding sites), it causes clustering of the Fc receptors, which stimulates the mast cell to release granules containing potent proinflammatory chemokines, in particular the vasoactive amines **histamine** and **serotonin**, as well as leukotrienes and cytokines such as IL-4 and IL-13 (Figure 4-4). These IgE-induced responses also initiate the strong inflammatory reactions associated with allergies and asthma (Box 4-2).

Many of the symptoms of infections caused by metazoal parasites (such as helminths, also known as parasitic worms) are traceable to elevated levels of IgE during infection. The release of mast cell granules in the vicinity of the intestinal wall may provoke an allergic response in the host that leads to ejection of the metazoal parasites from the intestinal or pulmonary mucosal sites. An interesting fact to ponder is that the human body evolved over millions of years to respond to worm infestations, with IgE functioning as part of that response. However, in recent times, particularly in developed countries, worms have been almost entirely eliminated from the human intestinal landscape. As such, in these more developed areas where metazoal infections are relatively uncommon, IgE is actually most often associated with noninfectious diseases such as allergies or asthma.

The most serious complication of the massive release of mast cell granules is anaphylaxis, which can rapidly kill a person. Is the rise in allergies and asthma seen in developed countries due to an immunological imbalance caused by elimination of a former enemy, thereby leaving the worm-oriented part of the specific and nonspecific defenses with nothing to do except cause trouble? Rest assured that this is not the start of a "bring back the worms" initiative, but it is interesting to contemplate the potential negative consequences of an abrupt change (in evolutionary terms) in our exposure to invaders that have been with us since we first appeared as a species.

Secretory Antibodies: Antibodies That Protect Mucosal Surfaces

IgA. In its monomeric form, IgA represents about 10 to 15% of the total serum antibody content. The role of IgA in blood and tissue is to aid in the clearance of antigen-antibody immune complexes from the blood. Because IgA monomer is a poor opsonin and activator, IgA in blood binds to the Fc receptor on immune effector cells, stimulating inflammatory responses and causing ADCC. By far the most important form of IgA, however, is the dimeric **secretory**

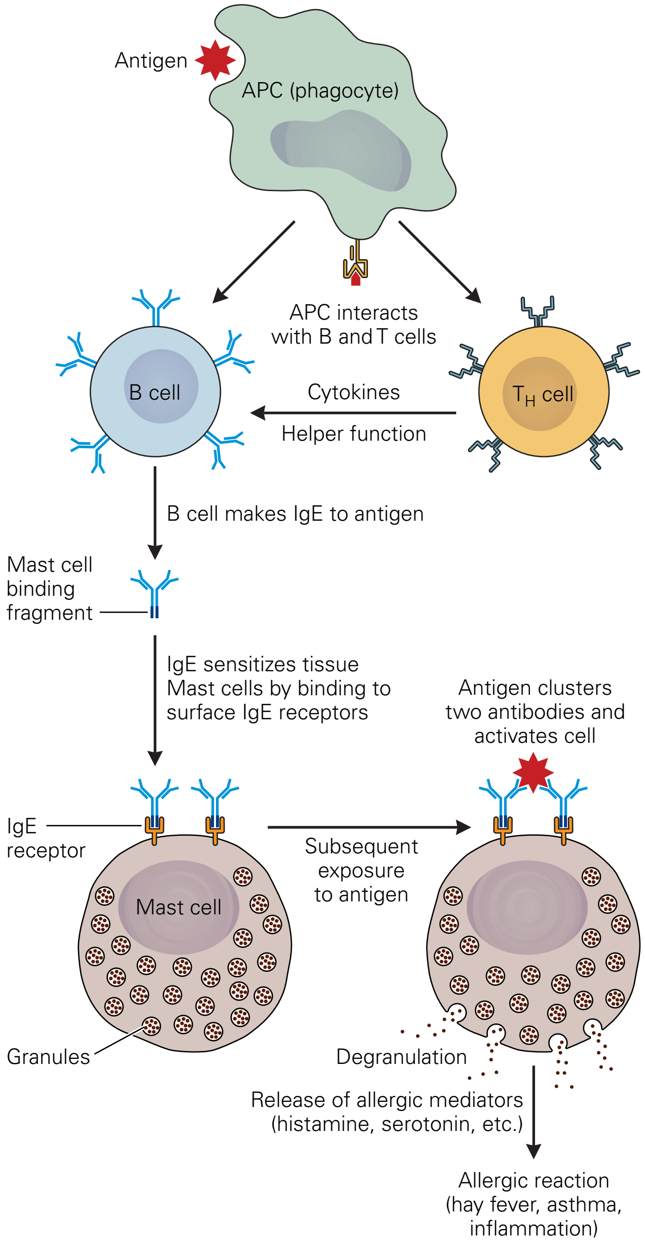
Antigen
APC (phagocyte)
APC interacts
with B and T cells
B cell
$\mathrm{T_H}$ cell
Cytokines
Helper function
B cell makes IgE to antigen
Mast cell
binding
fragment
IgE sensitizes tissue
Mast cells by binding to
surface IgE receptors
Antigen clusters
two antibodies and
activates cell
IgE
receptor
Mast cell
Subsequent
exposure
to antigen
Granules
Degranulation
Release of allergic mediators
(histamine, serotonin, etc.)
Allergic reaction
(hay fever, asthma,
inflammation)

Figure 4-4. Antibody-mediated mast cell degranulation. Antigen-mediated clustering of IgE bound to Fc receptors on mast cells triggers degranulation and release of allergic mediators, such as histamines, serotonin, vasoactive amines, and other inflammatory cytokines.

IgA (sIgA), the dominant antibody in mucosal secretions of the gastrointestinal, urogenital, and respiratory tracts, including tears, salivary glands, sweat, bile, colostrum, and milk.

Dimeric sIgA consists of two IgA antibody monomers joined end-to-end through disulfide bonds to a J-chain peptide and to another tightly bound peptide, called the **secretory piece** (Figure 4-1), acquired during transport out into the mucin layer (more on this later). The main role of sIgA is to attach to incoming microbes or toxic microbial components and trap them in the mucus layer, thus preventing them from reaching the epithelial surface. Because sIgA is also secreted into mother's milk, sIgA, like IgG, serves as an important protection against infection for infants who have not yet developed their own set of immune responses.

IgA is heavily glycosylated in the hinge region, which protects it from proteolysis. The secretory piece also helps protect the protease-sensitive sites in the H-chain from cleavage by bacterial and host proteases. Humans have two subtypes of IgA, IgA1 and IgA2. An interesting evolutionary development is the production of an IgA1-specific protease by a number of pathogenic bacteria (e.g., *Neisseria gonorrhoeae*), which is thought to have provided a selective pressure that resulted in human development of another gene encoding IgA2 that lacks the sites recognized by the IgA1 protease.

Pathogen and Toxin Neutralization by Antibodies

Just as sIgA antibodies are able to bind to microbes and prevent mucosal binding and entry into the host epithelium, both IgG and IgM antibodies bind to the surfaces of invading bacteria and viruses and prevent them from attaching to and entering target host cells (**neutralization of the pathogen**) (see Table 4-1). Antibodies are able to act as neutralizing agents because they are bulky molecules that can block interaction between microbial surface proteins and the receptors they recognize on host cells. Antibodies can also neutralize toxic proteins produced and secreted by bacteria by binding to the toxins and preventing them from binding to host cell surface receptors, thereby blocking their toxic effects (**toxin neutralization**). In addition, antibodies can directly neutralize the catalytic activities of secreted bacterial enzymes such as proteases, nucleases, or glycosidases that normally work to degrade host extracellular molecules and allow the pathogen to disseminate throughout the body.

An example of protective antibody neutralization of a bacterial toxin is the antibody response to *Corynebacterium diphtheriae*, the cause of **diphtheria**, a serious toxin-mediated disease of children. Corynebacteria often live innocuously in the upper respiratory tract, but when they become infected with a corynebacteriophage encoding the **diphtheria toxin** gene, they are then able to produce and secrete the diphtheria toxin, which enters cells lining the respiratory tract and bloodstream. Diphtheria toxin is one of the most potent bacterial toxins known and can kill many types of human cells. The action of the toxin in the throat is evident from a grayish or whitish "pseudo-membrane" patch that consists of dead epithelial cells and mucus. In some cases, this membrane can grow to the point at which it causes asphyxiation. If the toxin makes it through the bloodstream to the heart, it can cause death due to heart failure. The most effective protective response to infection by toxin-producing

Box 4-2.

The Dark Side of Adaptive Immunity: Allergy and Autoimmunity

Hypersensitivity is a set of undesirable, uncomfortable, and sometimes fatal reactions resulting from an overstimulation of inflammatory responses that cause host damage. Allergens that cause hypersensitivity include plant pollens, fungal spores, insect venom, animal dander, house dust mites, some foods, and certain chemicals (e.g., poison ivy, latex, jewelry, detergents, cosmetics). There are five types of hypersensitivity with differing times of onset and immune mediators:

Type I hypersensitivity occurs within minutes and is mediated through antigen binding and clustering of IgE-Fc receptors on mast cells and basophils, which causes release of histamine and vasoactive cytokines (as in Figure 4-4).

Type II hypersensitivity occurs within hours and is mediated through IgG- or IgM-receptor clustering on a target (infected) host cell, which the immune system then perceives as nonself and targets for attack by MAC by complement and/or ADCC by NK cells or macrophages.

Type III hypersensitivity occurs within hours and is mediated by IgG antibody binding to soluble, circulating antigens and forming a large immune complex that deposits at certain sites, such as joints, and causes local inflammation at those sites.

Type IV hypersensitivity is a delayed response that takes one to two days to manifest and is caused by $CD4^+$ Th1-memory-cell-mediated inflammatory responses to antigens or allergens.

Type V hypersensitivity (autoimmunity) is an IgG- or IgM-mediated immune response (similar to Type II) that is caused by antibodies binding to host cell antigens, which the immune system then perceives as nonself. This type of response sometimes occurs through molecular mimicry, in which a foreign antigen (such as a bacterium or bacterial product) shares structural features (epitopes) with certain host molecules, such that infection or exposure to the foreign antigen elicits antibodies against the common epitopes and thereby generates antibodies also against the host. Certain infections with superantigen-producing bacteria and viruses elicit strong nonspecific T cell or B cell activation and massive inflammatory responses that can also lead to autoimmunity.

C. diphtheriae, which is also the response elicited by the antidiphtheria vaccine, is the production of antibodies that bind to diphtheria toxin and prevent it from binding to and killing human cells (neutralization of the toxin).

Affinity and Avidity

A characteristic of antibody binding to antigens, which is of critical importance in assessing the effectiveness of the antibody, is the **avidity** of the antigen-binding site for the epitope it binds. **Avidity** is a combination of **affinity** (the strength of the binding interaction between an antigen-binding site and an epitope) and **valence** (the number of antigen-binding sites available for binding epitopes on an antigen). A single epitope can elicit a mixture of antibodies that vary considerably in affinity. This variation may arise because the body cannot know in advance what epitopes it will encounter, so it produces a variety of antibodies with differing antigen-binding sites, some of which will have a high affinity for a particular antigen. In fact, as the antibody response to an epitope develops, the B cells producing antibodies with the highest affinity will proliferate the most, and eventually those high-affinity antibodies will predominate.

High affinity is important, but it is not sufficient to ensure that an antibody bound to an epitope will retain its hold on the epitope. Because the binding between antigen-binding sites and epitopes is noncovalent, the interaction is reversible. Thus, there is an off-rate, as well as an on-rate, associated with antibody binding to an epitope. The importance of valence is that an antibody with a higher valence will be significantly less likely to detach from the antigen to which it is bound. If two antigen-binding sites of an antibody monomer bind to two adjacent epitopes on an antigen, the probability that both of them will detach at the same time is much lower than the probability that a single antigen-binding site will detach from its epitope. Thus, higher valence can improve the apparent strength of binding of an antibody to an epitope by orders of magnitude.

The differences in affinity and avidity between the first responders IgM/IgA and the adaptive IgG antibodies become important when one considers the roles that these different antibodies play in the immune response. Antibodies, such as IgM and sIgA, that appear early and have lower affinity and less specificity for the invading microbe need to have a higher valence number so that they can increase the avidity for the foreign antigen by binding more epitopes, up to 4 for sIgA or 10 for IgM. High avidity for an antigen is more effective at neutralizing microbes and toxins, blocking their binding and entry into cells. For IgM, high avidity significantly enhances the activation of complement and clustering of receptors to stimulate immune cells. Moreover, high avidity leads to agglutination that enhances opsonization of the microbes by phagocytic cells and clearance of the microbe from the circulation. Rapid clearance is a desirable feature because the longer antibody-antigen complexes remain in circulation, the more likely they are to deposit in the kidneys or other blood-filtering organs, where the complexes can activate complement and cause an inappropriate inflammatory response that damages the organ.

In contrast, IgG is a monomer and can bind only two epitopes, but because it is the product of an adaptive immune response, its low valency and hence low avidity is countered by its much higher affinity and specificity for the foreign antigen. Circulating IgG antibodies can not only bind very tightly to neutralize a specific microbe or toxin, but also mediate opsonization of the specific microbe through binding to Fc receptors on phagocytes. Antigen-bound IgG can also mediate complement activation through binding to a C1 complex.

Cytotoxic T Cells, Also Known as Cytotoxic T Lymphocytes (CTLs)

Cytotoxic T Lymphocytes: Critical Defense against Intracellular Pathogens

Like PMNs and NK cells (in ADCC), **cytotoxic T cells**, also called **CD8$^+$ T cells** or cytotoxic T lymphocytes (CTLs), kill infected host cells, but through a different mechanism. By entering cells, intracellular pathogens are protected from antibodies and complement. As such, killing infected host cells is often the only way to attack these invaders. CTLs and NK cells (in ADCC) are important parts of this defense response. The difference between CTLs and NK cells is that CTLs have **T cell receptors (TCRs)** that are specific to a particular epitope from a microbial antigen. Thus, while NK cells kill host cells infected with a variety of intracellular pathogens (see previous sections and chapter 3), CTLs kill only cells infected with a specific intracellular pathogen (see Figure 3-3).

CTLs have two mechanisms for killing infected cells. In the first, the CTL binds to a microbial antigen displayed on the surface of an infected cell using a TCR on its surface. (How the microbial antigen comes to be displayed on the cell surface will be described later.) This interaction then triggers the CTL to release granules containing two proteins, **perforin** (a pore-forming cytolysin that pokes holes in the host cell membrane) and proteolytic enzymes (**granzymes**) that enter the cell through the pore and trigger **programmed cell death (apoptosis)** in the infected cell (Figure 4-5). This type of attack kills the infected host cell but not the microbes. Instead, the released microbes are taken up by nearby activated macrophages that are better able to kill them. In the second mechanism, the CTL also releases a second pore-forming cytolysin from the granules, called **granulysin**. Granulysin is somewhat ineffective in lysing host cells, but it is very effective at killing bacteria. Presumably, granulysin kills bacteria the way perforin kills eukaryotic cells: by creating holes in the bacterial membranes and collapsing the proton motive force that bacteria use to gain energy. Additionally, perforin may also help deliver other yet-to-be-discovered antibacterial lysins or toxins into intracellular compartments of host cells.

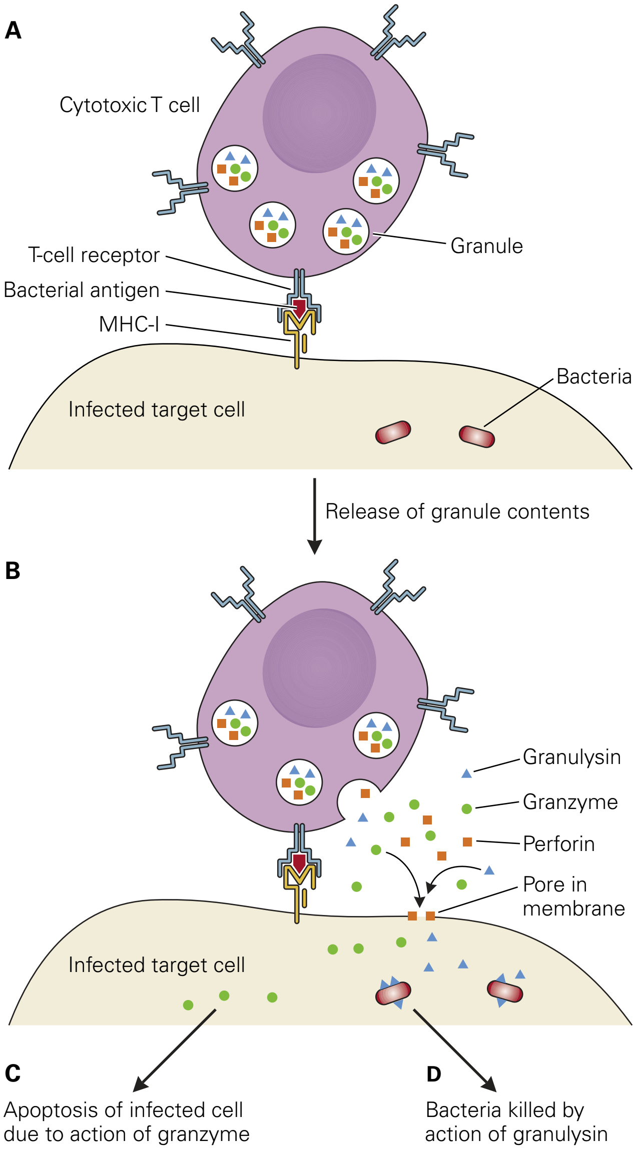

Figure 4-5. Cytotoxic T cell (CTL) action on infected host cells. Cytotoxic T cells (CTLs) mediate their action on infected cells through the release of perforin, granzyme, and granulysin from granules. **(A)** The cytotoxic T cell recognizes an infected target cell (e.g., macrophage) containing bacteria through T-cell receptor binding to a bacterial antigen displayed via MHC I. **(B)** The cytotoxic T cell releases granules containing perforin, granzymes, and granulysin. Perforin creates pores in the infected target cell membrane. Granzyme and granulysin enter the target cell through the pores created by perforin. **(C)** Granzyme triggers signaling pathways that lead to apoptosis of the infected cell. **(D)** Granulysin binds to and kills intracellular bacteria by creating pores in the bacterial membranes. The infected host cell then processes the degraded bacteria into antigens.

Antigen Presentation to the Immune System

Processing of Protein Antigens by Dendritic Cells

When microbes or their products first enter the body, professional phagocytes called **antigen-presenting cells (APCs)** engulf, process, and present antigens on their surfaces, which then direct other cells in the adaptive immune system to develop into cells with specific antibacterial activity. There are three types of APCs: dendritic cells (DCs), macrophages, and B cells. As described in chapter 3, macrophages and DCs are important players in the innate immune system, but they also serve as links to the adaptive immune system. Macrophages, as part of the innate immune system, are produced in an immature form (monocytes) and migrate through the bloodstream before moving into tissue where an infection is taking place. They help clear debris from dead human cells that may be circulating in blood or deposited in tissue. Macrophages also play a critical role in initiating and organizing the adaptive immune response.

DCs, like macrophages, initiate and organize the adaptive immune response, but because they are found mainly in localized areas of the dermis, the mucosal lining of the intestinal tract, and lymphoid tissue, they are the first APCs on the scene to process microbial antigens and stimulate the adaptive immune response. B cells can also function as APCs, albeit not as efficiently as macrophages or DCs. In addition to producing and secreting soluble antibodies, B cells also produce **membrane immunoglobulins (mIg)** that they display on their surfaces. When the mIg captures an antigen, the mIg-antigen complex is internalized and the antigen is processed and presented to the T helper cells.

Peptide Antigens. Figure 4-6 shows an overview of how APCs process protein antigens and display the resulting peptide epitopes on their surfaces bound to a protein complex called the **major histocompatibility complex (MHC)**. Two main classes of MHC molecules, **MHC I** and **MHC II**, form complexes with peptide epitopes (Figure 4-7). MHC I molecules are produced by all nucleated cells in the body, while only professional phagocytic APCs, such as DCs, macrophages, B cells, and certain activated epithelial cells, produce MHC II molecules in addition to MHC I molecules. In the case of peptide antigens, the type of MHC used to display the epitope determines the type of immune response that will be elicited (more on this later).

How does an APC decide whether to display an epitope on MHC I or MHC II? This question has received a great deal of attention because understanding what leads to each type of presentation is critical for developing effective vaccines. From cumulative data so far, some basic rules have emerged. Intracellular pathogens such as viruses and some bacteria, particularly those that can enter the cytoplasm or nucleus of an APC, are most likely to elicit an MHC I display of the epitopes (Figure 4-6A). Additionally, large particulate or aggregated antigens that do not escape the phagocytic or pinocytic vesicle seem to elicit primarily an MHC I-linked display. By contrast, epitopes processed from soluble antigens, such as peptides or proteins secreted from bacteria or those exposed on the surface of extracellular pathogens, are displayed almost exclusively by MHC II (Figure 4-6B). Thus, if one wishes to use peptides to elicit a CTL response, it is necessary to present these peptides in particulate form (e.g., bound up in a large immune complex, or to deliver them directly into the cytosol to ensure their processing will occur via the MHC I pathway). Alternatively, these peptides could be expressed intracellularly using mammalian expression vectors (more on this in chapter 17).

Differences in immunogenicity have important practical consequences. For example, it is now possible to produce epitope-sized peptides synthetically. Peptides are not only much cheaper to produce than full-length proteins, which must be purified through time-consuming biochemical procedures, but they also make it possible to target a specific antibody or CTL response toward one or more defined regions of an antigen. Epitopes differ in the way they are recognized by the immune system, and only a small subset of the potential epitopes present in any given antigen will elicit an immune response. Epitopes that are processed and presented on the surface of APCs bound as MHC complexes, which are then recognized by T cell receptors on Th cells, are referred to as **T cell epitopes**. T cell epitopes that bind to MHC I complexes are typically 8 to 11 amino acid residues in length, whereas T cell epitopes that bind to MHC II complexes are 13 to 17 amino acid residues in length. Epitopes that are recognized by antibodies expressed on the surface of the B cells are called **B cell epitopes**. B cell epitopes vary from 5 to 10 amino acids in length and are found on the exposed surface of the native conformation of the antigen (i.e., they are not processed by APCs). B cell epitopes can elicit strong antibody responses, while T cell epitopes can also elicit strong cell-mediated CTL responses.

Directing the adaptive immune response toward particular epitopes is important because not all immunogenic epitopes elicit protective responses. Some immunogenic epitopes, for example, are buried within a folded protein and are therefore inaccessible to the antibody or are expressed inside a microbe and are thus not exposed to circulating antibodies. Eliciting an antibody response to such an epitope is useless because the antibody will not be able to bind and neutralize the antigen or enhance its opsonization. Furthermore, some microbial proteins have regions that vary considerably from one strain of microbe to another. Antibodies or cytotoxic T cells that recognize highly variable regions of microbial proteins will only be useful against a limited number of strains. A better strategy is to target regions of microbial proteins that are exposed and highly conserved (i.e., found in all strains of the microbe). Using peptide epitopes as vaccines makes it possible to program a

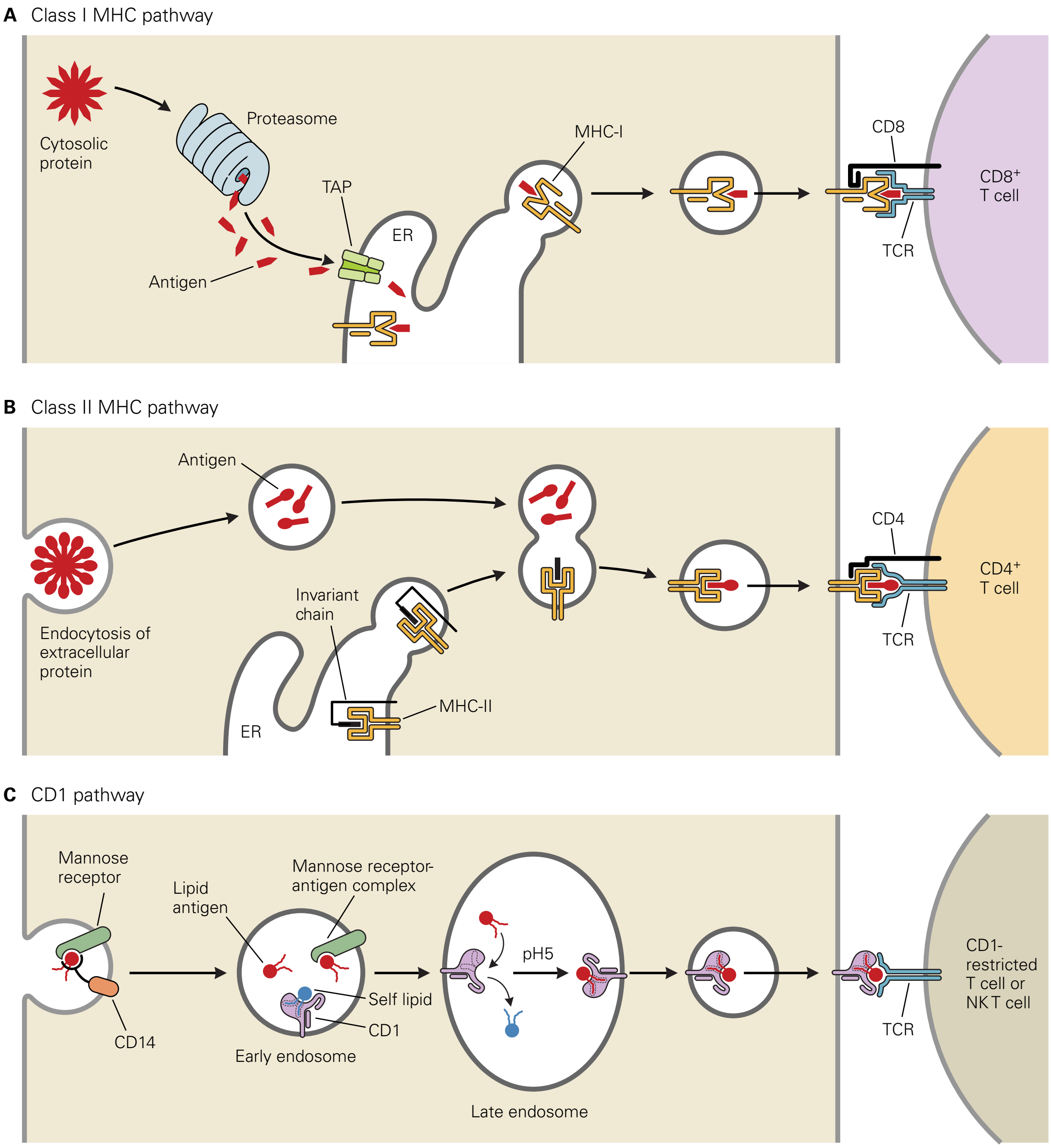
A Class I MHC pathway
Cytosolic protein
Proteasome
TAP
Antigen
ER
MHC-I
CD8
CD8+ T cell
TCR
B Class II MHC pathway
Antigen
Endocytosis of extracellular protein
Invariant chain
ER
MHC-II
CD4
CD4+ T cell
TCR
C CD1 pathway
Mannose receptor
Lipid antigen
Mannose receptor-antigen complex
CD14
Self lipid
CD1
Early endosome
pH5
Late endosome
TCR
CD1-restricted T cell or NKT cell

Figure 4-6. MHC I, MHC II, and CD1 pathways of antigen processing by APCs and presentation to T cells leads to activation and increased proliferation of T cells. (A) In the MHC I pathway, protein antigens present in the cytosol are processed by the proteasome, and the resulting peptides are transported to the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP). In the endosome, the peptide antigens are further processed by an endosomal protease into smaller peptide epitopes, which then bind to MHC I, and the complex traffics to the cell surface, where it binds to the T cell receptor (TCR) and CD8 on the surface of CD8$^+$ T cells. **(B)** In the class II MHC pathway, extracellular protein antigens are endocytosed into late endosomal and lysosomal vesicles, where they are processed into peptides that displace the invariant chain of the MHC II molecule. An accessory protein, called H-2M in mice (HLA-DM in humans), facilitates the displacement of the invariant chain with the peptide epitope. The peptide epitope-MHC II complex is then transported to the cell surface, where the complex binds to the T cell receptor (TCR) and CD4 on the surface of CD4$^+$ T cells. **(C)** The CD1 antigen presentation pathway. Uptake of foreign glycolipid or lipid antigens occurs through multiple pathways. Glycolipid antigens bind to APCs via pattern recognition molecules such as CD14 and the mannose receptor. The low-density lipoprotein receptor (LDLR) can bind to the lipoprotein transporter ApoE. The mannose receptor can also mediate the uptake and trafficking of such lipid or glycolipid antigens through the endosomal pathway where, at acidic pH, the lipid portions of the glycolipid antigens are released and displace the self-lipids at the binding cleft of the CD1 molecule. Accessory proteins, called saposins, and possibly CD1e help process the lipid antigen and displace the self-lipid on the CD1 with the lipid antigen. An ER protein, called microsomal triglyceride transfer protein (MTP), facilitates the loading of the self-lipids onto CD1. The antigen-CD1 complex then traffics to the cell surface, where it is recognized by the CD1-specific T cell receptor. CD1-mediated antigen presentation occurs through TCRs in both αβ T cells and γδ T cells.

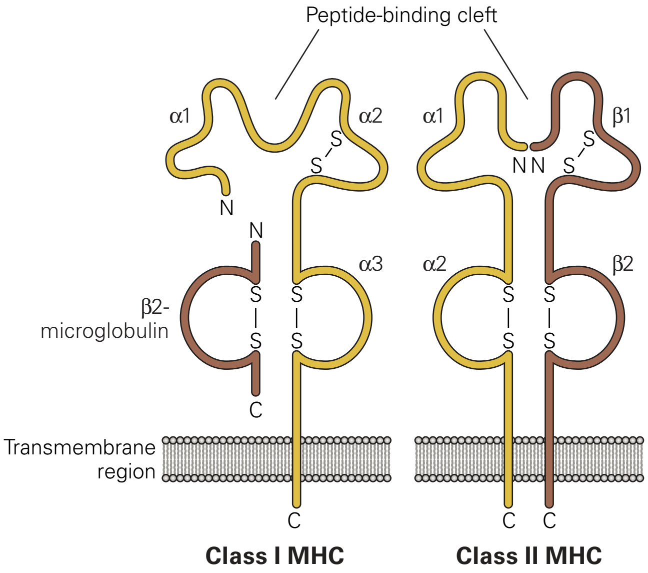

Figure 4-7. Major histocompatibility complexes I and II. Shown are the structures of major histocompatibility complex I (MHC I) and II (MHC II) proteins, which bind to peptide antigens and present them to T cell receptors (TCRs) on the surface of Th1 and Th2 cells, respectively. NOTE: In humans, the corresponding MHC molecules are called human leukocyte antigens (HLA).

specific immune response directed toward conserved epitopes that are exposed on the surface of the antigen. We will return to this concept in chapter 17.

Other Kinds of Macromolecular Antigens. Unfortunately, many of the bacterial surface antigens recognized by the immune system are lipid, carbohydrate, or lipid-carbohydrate combinations. Gram-negative LPS and Gram-positive LTA and PG are excellent examples. In the past, immunologists and vaccinologists have focused almost exclusively on peptide antigens because they are easier to characterize than carbohydrate or lipid-containing molecules and because peptides elicit a strong immune response. Peptide antigens consist of different amino acids linked by a single type of bond, the peptide bond, which can be readily processed into defined epitopes. Carbohydrate oligomers, by contrast, can be linked by any of 12 types of glycosidic linkages. Lipids also contain more than a single type of linkage. Because of the great diversity of carbohydrate, lipid, and lipid-carbohydrate molecules, it is not surprising that the mechanism for how these carbohydrate and lipid antigens are processed has been largely neglected until recently.

A major breakthrough in this area came with the discovery of **CD1 molecules**. *Mycobacterium tuberculosis*, the cause of tuberculosis, provides a cornucopia of lipid-saccharide and lipid-peptide antigens not found in most other bacteria, which enabled researchers to identify the first CD1 complexes that were bound to lipid or glycolipid antigens. Since then, five forms of human CD1 have been found: CD1a through CD1e. The steps in processing and displaying lipid antigens appears to be similar to those for peptide antigens (Figure 4-6C), with CD1 taking the place of the MHC I molecule for presentation of antigens to CD1-specific CTLs (for CD1a, CD1b, and CD1c) or NK cells (for CD1d). CD1e is the only isoform that does not appear to be expressed on the surface of DCs, and it is suspected that it might play a role in antigen processing, rather than in display of the antigen. CD1 is related, at the amino acid sequence and overall domain structure level, to MHC I, but has obviously diverged during evolution. CD1 proteins have deeper and larger binding cavities to accommodate the hydrophobic alkyl chains of lipid antigens. The more hydrophilic head group of the lipid antigen faces outward and interacts with the TCR on the CTLs and NK cells.

Interaction between APCs and T Cells: The T-Cell-Dependent Response

When an APC displays a particular MHC I-epitope or MHC II-epitope complex on its surface, only a small number of the vast pool of available immature precursor T cells will have a TCR capable of recognizing that particular MHC-epitope combination and stimulating the signals needed to induce proliferation and production of cytokines (Figure 4-8). All T cells express CD3 complexes (comprised of γ, δ, and ε chains) and TCR complexes (comprised of α, β, and two ζ chains). The CD3 and ζ chains are invariant, but the large repertoire of TCRs with surface receptors that recognize a wide variety of different MHC-bound epitopes is generated through variation of the αβ chains, which undergo V(D)J recombination during T cell development in the thymus (analogous to the process mentioned earlier that leads to antibody diversity).

Antigen presentation and binding to the TCR stimulates the T cell to become either a CTL or a T helper (Th) cell (Figure 4-9). Display of intracellularly derived peptide epitopes on MHC I allows the APC to activate and stimulate the proliferation of CTLs. CTLs are distinguished by the presence of **CD8 molecules** on their surfaces (hence the name CD8$^+$ cytotoxic T cells). The CD8 molecules, along with the T cell receptor (TCR), are responsible for recognizing only antigen-bound MHC I complexes on the APC. CD8 binds to MHC I and stabilizes the interaction between epitope-bound MHC I and the TCR. CTLs are particularly well equipped to recognize infected cells because virtually all cells of the body produce MHC I. If a cell is infected, it displays epitopes from the invading microbe on its surface. Binding of a CTL to the surface of an infected cell causes the CTL to release cytolytic or apoptotic proteins that can kill the infected cell, as described earlier (see Figure 4-5). In addition, as described in chapter 3, NK cells also use the amount of MHC I on cell surfaces as an indicator of cell health, because infected cells generally express less MHC I than normal cells.

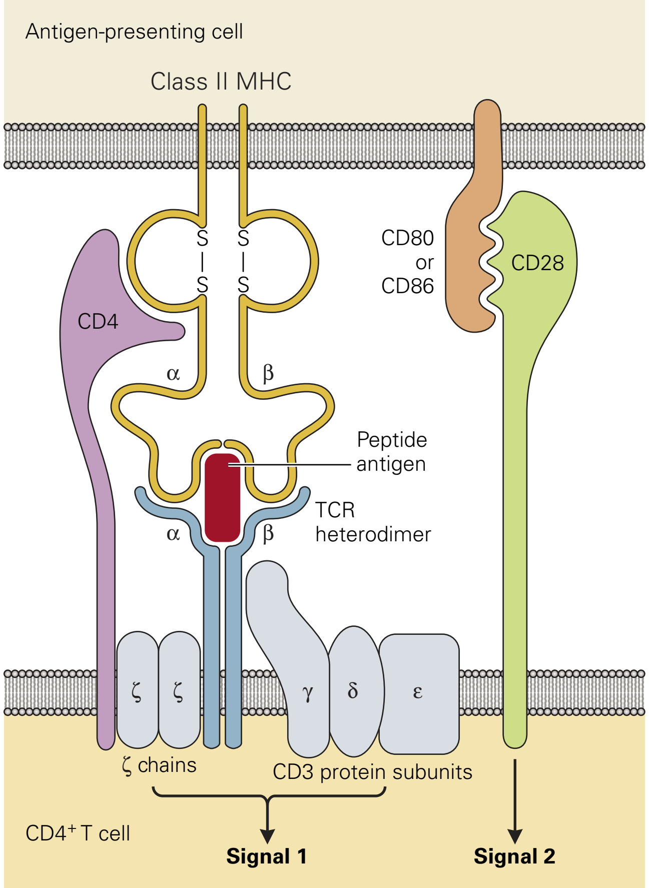

Figure 4-8. The T cell receptor (TCR) complex. Shown is a schematic illustration of the complex formed between the epitope-MHC II complex on the APC and the CD3-TCR complex on the CD4$^+$ Th cell. Note: Similar complex formation occurs for MHC I recognition by CD3-CD8$^+$ T cells. CD4 and CD8 are expressed on two different types of αβ T cells, where CD4 recognizes MHC II complexes and CD8 recognizes MHC I complexes on APCs. Activation of the T cells requires stimulation by two signals: signal 1 occurs through specific interaction of the epitope-MHC II with the CD4-TCR and CD3 complexes and signal 2 occurs upon costimulation through the CD28 binding to CD80 or CD86 on the APC.

In contrast, if an epitope is presented by the APC in complex with MHC II rather than MHC I, a different class of T cells, T helper (Th) cells, is stimulated. Complex formation between TCRs and coreceptor **CD4 molecules** on the surface of precursor Th cells (called CD4$^+$ Th0 cells) with epitope-bound MHC II molecules on the surface of APCs leads to production of IL-2 and then IL-4 by the Th0 cell and proliferation and maturation. Proliferating Th cells come in two types: Th1 and Th2 cells. These Th cells influence a variety of immune responses through release of cytokines, and how they do this is a very active area of research in the field of immunology.

Other proteins on the surface of the APC and the Th cell, called costimulatory molecules (e.g., CD54, CD11a/CD18, CD58, CD2), must also interact to make the binding between the APC and the Th cell tight enough to stimulate the APC to release cytokines that stimulate the Th cell to proliferate and become activated. **Th1 cells** are stimulated to proliferate by IL-12 and IL-2 released by APCs, which causes the Th1 cells to release more IL-2 (positive feedback), IFN-γ, and TGF-β. Recognition of MHC II-epitope by CD4$^+$ Th1 cells also stimulates the production and release of IL-2 and IFN-γ, which in turn activate macrophages. Th1-cell-mediated responses lead to cellular immunity, which is most effective against intracellular pathogens. The necessity for contacts between different surface proteins of the APC and the Th cell helps ensure that only specific binding of an MHC-epitope complex to its cognate TCR will result in Th cell activation.

Th2 cells are stimulated to proliferate by IL-4, which causes the cells to release more IL-4 (positive feedback), as well as other cytokines (IL-5, IL-9, IL-10, and IL-13), which modulate other immune cells. MHC II-epitope stimulation of CD4$^+$ Th2 cells stimulates naïve B cells to proliferate into B cells and mature into antibody-producing plasma cells (i.e., humoral immunity) and memory B cells. IL-4 also stimulates B cells to produce IgE antibodies, which in turn stimulate mast cells to release their cytokines (histamine, serotonin, and leukotrienes). The Th2 response is most effective against extracellular pathogens.

Once activated, T cells begin to proliferate, with most of the resulting effector Th cells becoming involved in combating the invading microbes. A few of the T cells, however, become quiescent **memory T cells**. Memory T cells persist for long periods in the body. They are generally present in higher numbers than naïve T cells with the same TCR, and they are more easily stimulated to proliferate and produce stronger cytokine responses when they encounter their specific epitopes on APCs during subsequent infections. Memory T cells thus allow the body to respond to a second encounter with a particular microbial invader much faster and more strongly than it did after the initial encounter.

Some bacteria and viruses produce proteins called **superantigens** that interfere with this highly specific progression of events in an interesting way that will be described in greater detail in chapter 12. Superantigens force a close association between APCs and T cells through abnormal association of MHC and the TCRs without a matched antigen. Normally, only a small fraction of T cells will interact with APCs presenting antigens on their surface. Superantigens, on the other hand, can cause up to 20% of T cells to participate in such interactions. As the cytokine signaling begins, cytokines are produced at higher levels than normal and this overreaction can trigger shock. The term "septic shock" is sometimes used to describe this phenomenon; however, as described in chapter 3, LPS or other bacterial surface components usually initiate what is considered septic shock. The term "T-cell-mediated shock" or "toxic shock" might be

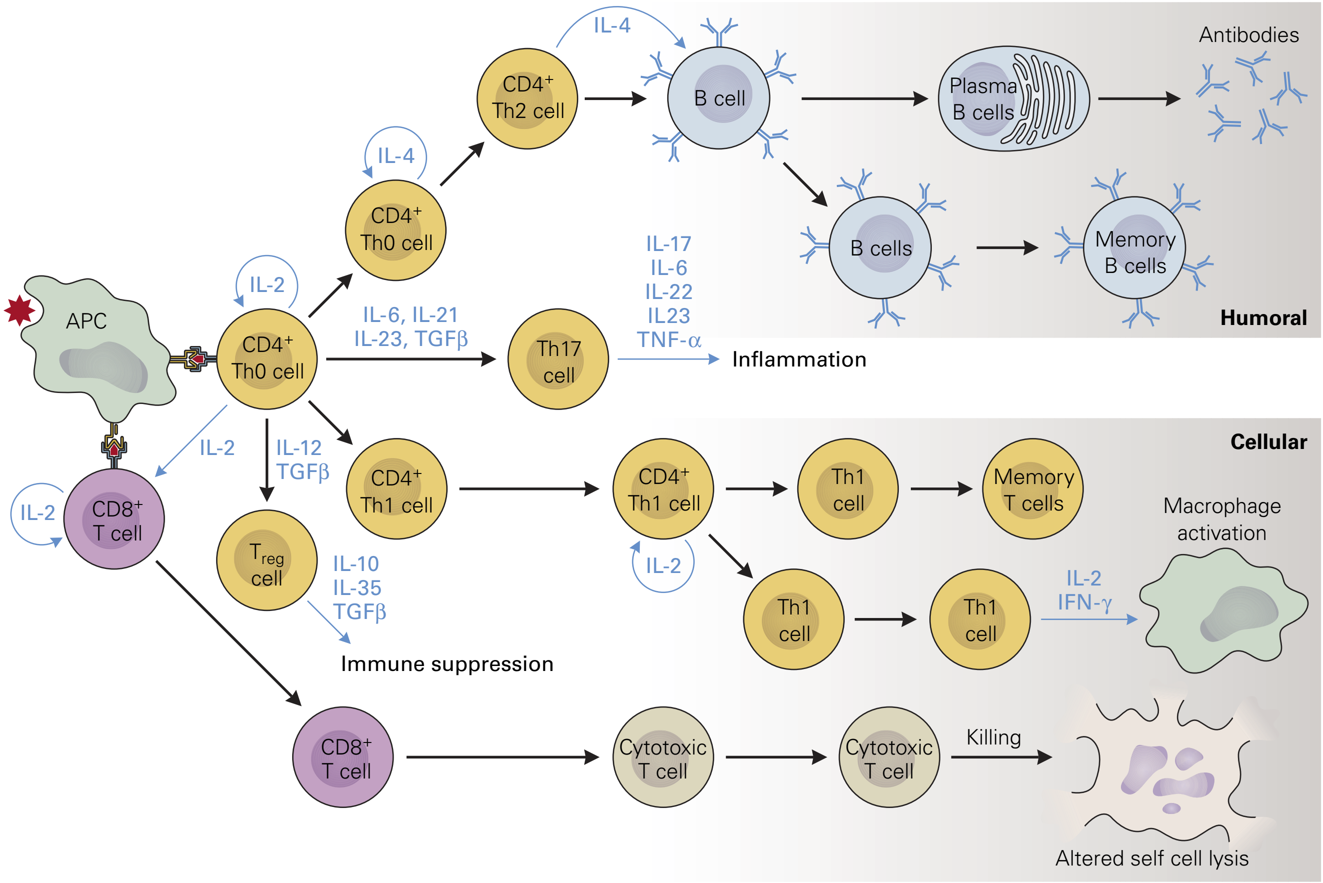
IL-4
CD4+ Th2 cell
B cell
Plasma B cells
Antibodies
IL-4
CD4+ Th0 cell
B cells
Memory B cells
IL-2
APC
CD4+ Th0 cell
IL-6, IL-21
IL-23, TGFβ
Th17 cell
IL-17
IL-6
IL-22
IL23
TNF-α
Inflammation
Humoral
Cellular
IL-2
IL-12
TGFβ
CD4+ Th1 cell
CD4+ Th1 cell
Th1 cell
Memory T cells
IL-2
CD8+ T cell
Treg cell
IL-10
IL-35
TGFβ
IL-2
Macrophage activation
Th1 cell
Th1 cell
IL-2
IFN-γ
Immune suppression
CD8+ T cell
Cytotoxic T cell
Cytotoxic T cell
Killing
Altered self cell lysis

Figure 4-9. T-cell-mediated immunity and memory. Characteristics of the antigen (Ag) determine whether the antigen is presented via APCs through MHC I complexes or through MHC II complexes. Ag-MHCI complexes bind to TCRs on CD8$^+$ T cells that trigger the CTL response. Ag-MHC II complexes that bind to TCRs on CD4$^+$ Th0 cells trigger IL-2 cytokine production and maturation into CD4$^+$ Th1 cells, leading to cellular immunity or, in the subsequent presence of IL-4 into CD4$^+$ Th2 cells, leading to stimulation of the antibody-producing B cell response. Cytokines and the presence of Th17 cells help direct the type of cell-mediated immunity and memory that occurs. Treg cells help control the immune response, especially once the pathogen has been cleared from the system.

more appropriate to describe the shock initiated by superantigens, to distinguish it from septic shock.

Th-(Th1/Th2/Th17)-Cell-Mediated Immunity

A paradigm for the process by which T helper (Th) cells influence the development of different types of immunity (such as cytotoxic T cells, activated macrophages, antibodies, and the IgE response) has emerged in recent years. The simplest form of this paradigm is the Th1/Th2 cell model, in which there are two subtypes, Th1 and Th2, that control the development of acquired immunity (Figure 4-9). Both Th1 and Th2 cells are descended from the same cell type, Th0. The decision to produce Th1 cells is triggered in part by PMN production of IL-12, which stimulates NK cells to produce IFN-γ, which in turn stimulates Th0 cells to differentiate into the Th1 form. Extracellular antigens stimulate differentiation of Th0 cells to develop into Th2 cells. IL-4 is required for Th2 differentiation and is later produced by mature Th2 cells. Cytokines produced by Th1 cells, such as IFN-γ, or by Th2 cells, such as IL-4, induce a positive feedback loop that leads to further differentiation of Th0 cells into Th1 or Th2 cells, respectively, and inhibits production of the other cell type. Later in the course of combating infection, the balance between Th1 and Th2 differentiation is eventually restored.

A third, more recently discovered pathway is the Th17 pathway (Figure 4-9), so called because Th17 cells are CD4$^+$ Th cells and CD8$^+$ cytotoxic T cells that produce IL-17. The main protective role of the Th17 pathway is thought to stimulate proinflammatory reactions that lead to recruitment of PMNs and neutrophils in tissues. Th17 cells also produce other cytokines, including IL-22, which induces antimicrobial peptide production by epithelial cells that protect against extracellular bacteria and fungi.

Th2 cells activate eosinophils and stimulate B cells to produce antibodies of the IgG1 class (the most effective opsonizing antibodies) as well as antibodies of the IgE class (the class associated with the allergic or antimetazoan response). Th2 cells also produce IL-10 and IL-13, cytokines that downregulate some cells of the immune system. Thus, the Th1-type response is the most desirable type of response to viral and intracellular bacterial infections. The Th2-type response produces the more effective opsonizing antibodies, which are important for clearing extracellular bacterial pathogens. An example of the type of Th1 or Th2 response that is elicited and its importance in disease outcome is illustrated in Box 4-3.

A fourth pathway that contributes to T cell regulation, the so-called Treg pathway (or Th3 pathway), has now been identified. The Treg cells produce additional regulatory compounds that control the differentiation of Th0 cells. Treg cells maintain tolerance to self-antigens and modulate autoimmune responses and serve to suppress other immune cells, particularly once invading pathogens have been cleared from the body. Cytokines produced by Treg cells can direct the outcome of an immune response, tipping the balance between one dominated by a Th1 or a Th2 pathway (as in Box 4-3).

Production of Antibodies by B Cells

Two signals are required to activate B cells to differentiate into plasma cells and produce antibodies (Figure 4-10). This failsafe costimulatory mechanism makes sense, considering the dire consequences of not strictly controlling antibody production. One mechanism that activates B cells is the associated Th cells that mature in the thymus gland (**thymus-dependent antigens**). Resting B cells are not dividing, but they do express a single type of membrane immunoglobulin (mIg) on their surfaces. There are an enormous number ($>10^{10}$) of resting B cells displaying mIg molecules with different antigen specificities on their surfaces! The mIg molecules interact with a membrane-bound transducer protein to form the **B cell receptor** (**BCR**). When an antigen binds to the Fab regions of two mIg molecules, linking them together, a signal is sent to the B cell that can lead to cell proliferation, if a second signal is detected. The second signal is generated by endocytosis and presentation of the antigen on the MHC II-antigen complexes on the surface of the same B cell. The use of MHC II helps B cells find the activated Th cells that are displaying TCRs specific to the displayed antigen, which ensures specific responses to a particular antigen. Binding of a Th cell to the B cell involves a set of multiprotein contacts similar to those that occur between other APCs and T cells (Figure 4-10). This binding causes the Th cell to produce additional surface receptors that lead to even tighter binding between the antigen-stimulated B cell and the Th cell. This tight binding stimulates the B cell to produce and display cytokine receptors and the Th cell to produce cytokines, which in turn stimulate the B cell to proliferate and differentiate into the mature form of B cells, called plasma cells, that secrete antibodies.

Box 4-3.

The Th1/Th2 Response and the Outcome of Leprosy and Tuberculosis

Some people who contract tuberculosis, caused by *Mycobacterium tuberculosis,* or leprosy, caused by *Mycobacterium leprae,* develop a systemic form of the disease. In the case of tuberculosis this is called miliary tuberculosis, while in the case of leprosy this is called lepromatous leprosy. Other people develop a more localized form of the disease and, although there is damage in the area of infection, bacteria do not spread so readily to internal organs. What causes some people to develop the localized type of infection and others to develop the more dangerous systemic form of infection? What both *M. tuberculosis* and *M. leprae* have in common is that they enter and replicate in macrophages. An explanation based on the Th1/Th2 response has been proposed and seems to be supported by the evidence so far.

In people who are able to elicit a robust Th1-type immune response that leads to generation of activated macrophages, the numbers of invading bacteria are greatly reduced and the infection tends to be localized. In people who do not mount a strong Th1-type immune response, but instead mount a Th2-type response, which does not contribute to activation of macrophages but rather to production of antibodies, the bacteria reach high numbers in macrophages and subsequently disseminate throughout the body. Thus, balance between the Th1- and Th2-initiated paths of immune response to infection has critical practical implications for patients. This is one of many reasons why immunologists are trying to understand the complex interplay of cytokines that controls this crucial decision. Obviously, understanding this decision is crucial to the development of effective vaccines and therapies. Indeed, in cases for which a vaccine might not be available, development of pharmaceutical drugs capable of directing the Th1-Th2 decision branching could potentially manipulate the immune response to favor the more effective one against a particular infection. Recent evidence suggests that Treg and Th17 cells play important roles in directing the Th1-Th2 decision branching.

Source:

Modlin RL. 1994. Th1-Th2 paradigm: insights from leprosy. *J Invest Dermatol* **102:**828–832.

Sadhu S, Khaitan BK, Joshi B, Sengupta U, Nautiyal AK, Mitra DK. 2016. Reciprocity between regulatory T cells and Th17 cells: relevance to polarized immunity in leprosy. *PLoS Negl Trop Dis* **10:**e0004338.

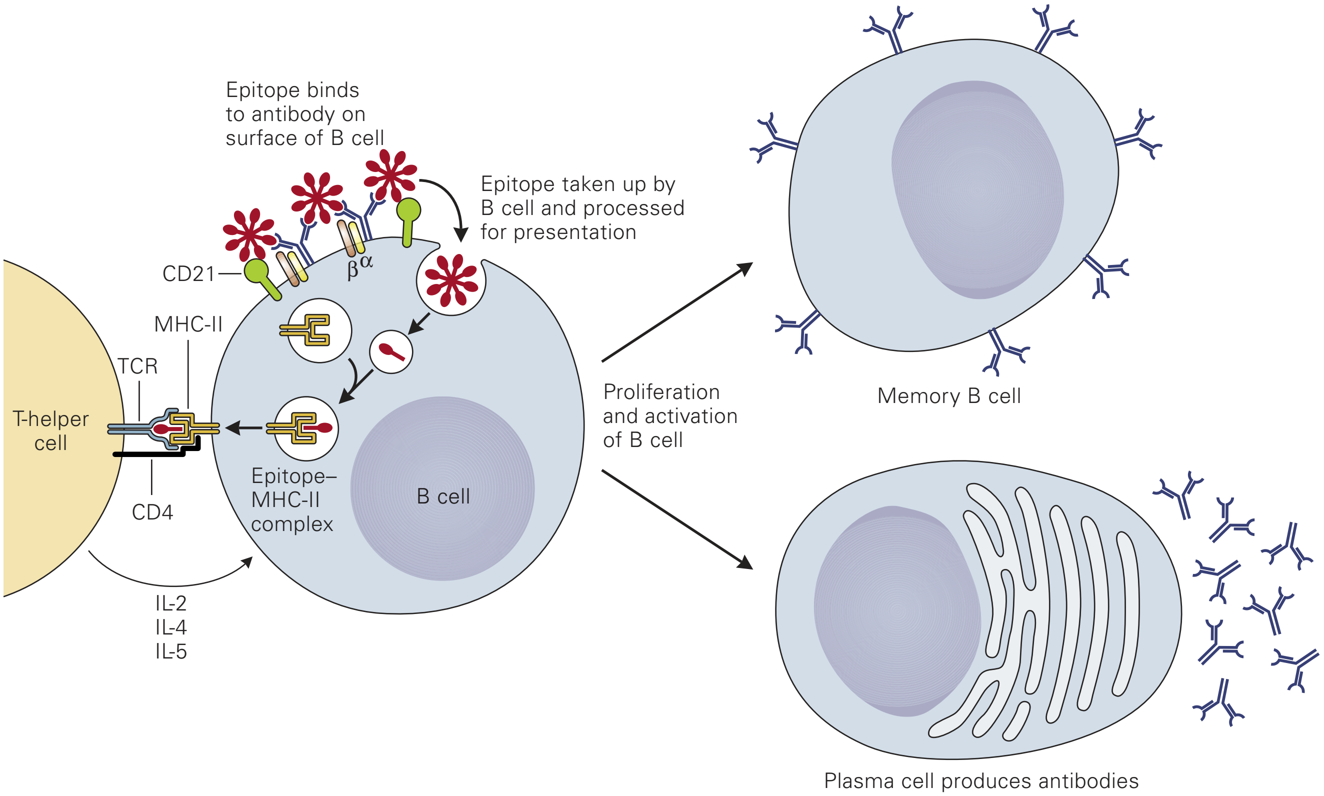

Figure 4-10. The Th2 cell binds and activates B cells presenting the same epitope on their MHC II. The B cell receptor complex is comprised of membrane-bound immunoglobulin (mIg), which can be of isotype IgM, IgD, IgA, IgG2, or IgE, the signaling mediator proteins Igα and Igβ, and the CD21 complement receptor that recognizes iC3b and promotes B cell activation. For simplicity, many of the other proteins involved in binding and activating the B cell (e.g., CD54, CD11a/CD18, CD2, CD58, CD80/70, CD28) are omitted. Stimulation of B cells through interactions of CD40 on the B cells with CD40 ligand expressed by Th cells and through cytokines produced by Th cells results in proliferation of the B cells and conversion into antibody producing cells, called plasma cells. A fraction of the activated B cells become memory B cells.

A fraction of activated B cells become quiescent memory B cells. In subsequent encounters with the same antigen and again with memory Th cells against that same antigen, the memory B cells respond with a much shorter lag period and produce more antibodies for a longer period of time than in the primary response of the naïve Th and B cells. In addition, this secondary response produces antibodies with higher affinity, primarily of the IgG isotype.

Links between the Innate and Adaptive Defense Systems

Treating the innate and adaptive defense systems as separate subjects in separate chapters makes pedagogical sense because it is generally easier to understand complex systems by first separating them into their constituent components. However, it is important to note that for the human body to have the innate defenses acting independently of the adaptive defenses would be counterproductive, because the two types of defense need to act synergistically. Some examples of links are **cytokines** and **chemokines**, which are produced by and act on both types of immune responses; PMNs that serve as first responders and contribute through cytokine production to the Th1/Th2 decision-making process; DCs that opsonize pathogens and present the processed antigens to the adaptive immune cells; and antibodies that are made as an adaptive response but, when bound to bacterial cells, activate one arm of the complement cascade (i.e., the classical pathway).

We now add yet another link: the interaction between natural killer cells and B cells. It may be simply a coincidence that natural killer (NK) cells have a number of similarities, morphologically and cytokinologically, to T cells. Because T cells interact with B cells on other planes of existence, it is somewhat satisfying to learn that NK cells interact with B cells, too. Briefly, NK cells can stimulate B cells to proliferate (as T helper cells do) but by some factor that is not one of the known cytokines. B cells in turn induce NK cells to secrete more IFN-γ, which, as we have already seen, is an important factor in macrophage activation.

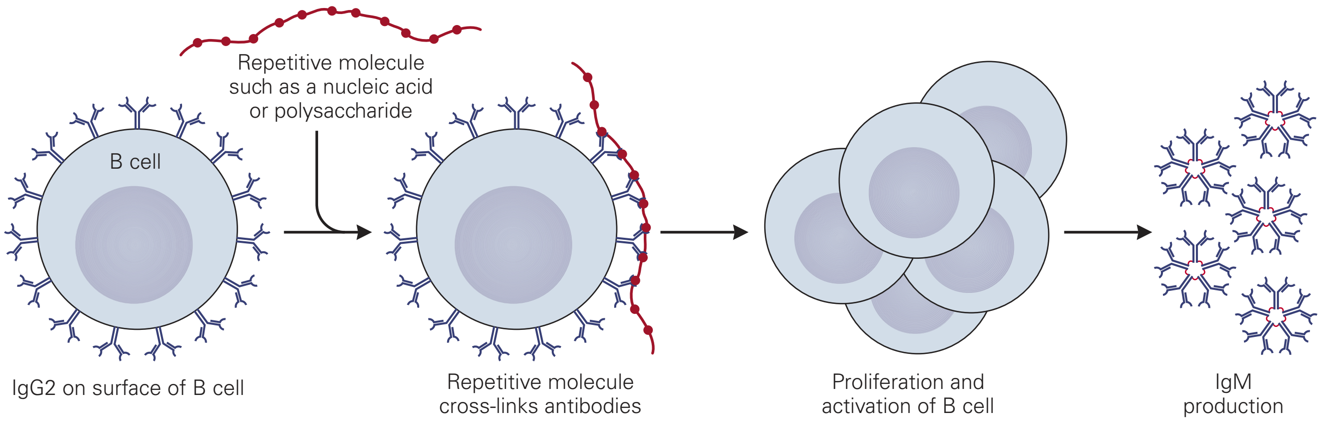
Repetitive molecule such as a nucleic acid or polysaccharide
B cell
IgG2 on surface of B cell
Repetitive molecule cross-links antibodies
Proliferation and activation of B cell
IgM production

Figure 4-11. T-cell-independent production of antibodies. Macromolecules with highly repetitive structures, such as carbohydrates (polysaccharides, capsules) and nucleic acids, are able to directly stimulate naïve B cells to proliferate, mature, and produce antibodies through antigen-mediated clustering of IgG2 and IgM antibodies on the surface of the B cells.

T-Cell-Independent Antibody Responses

In addition to the CD1-dependent T cell pathway discussed earlier (Figure 4-6C), nonpeptide antigens such as polysaccharides (e.g., bacterial capsule components), nucleic acids (e.g., bacterial DNA or RNA), and to a lesser extent lipids and glycolipids (e.g., bacterial membrane components) can also stimulate **T-cell-independent antibody responses.** T-cell-independent antigens provoke an antibody response by directly interacting with B cells (Figure 4-11), without the involvement of T cells. Polysaccharides are a well-studied class of T-cell-independent antigens. Those B cells displaying mIg with affinity for the sugar repeat units will bind to the polysaccharide chain. Because these epitopes are repeated many times, numerous mIgs and BCRs are linked together, which causes them to cluster and activate cellular signaling pathways. This acts as one signal to stimulate the B cells to proliferate and differentiate into mature antibody-producing plasma cells. A second signal, such as binding of cytokines produced at the infection site, is also required to initiate the T-cell-independent response.

The T-cell-independent response is particularly important for protection against bacterial pathogens that can avoid phagocytosis by covering themselves with an exopolysaccharide layer (capsule), which is not effectively opsonized through coating with C3b. Such bacterial pathogens are only ingested and killed by phagocytes if antibodies that bind to capsular antigens are elicited and act as opsonins.

Although the T-cell-independent response provides protection against capsule-producing bacteria, it has some important drawbacks. First, the antibody response elicited by T-cell-independent antigens is not as strong as the T-cell-dependent response. It is also not long-lasting, because no memory B cells are developed. Second, the main antibodies elicited by T-cell-independent antigens are IgM and IgG2. IgG2 does not opsonize and IgM does so less effectively than IgG1 and IgG3 (Table 4-1). Third, infants do not mount a T-cell-independent response. Polysaccharides and lipids can elicit an immune response in children and adults, but not in infants under the age of two years. Thus, the ability to respond to T-cell-independent antigens, such as capsule exopolysaccharide, is acquired after birth. This is an important consideration because it means that vaccines consisting of T-cell-independent antigens are not effective until an infant has become old enough to respond to these antigens. Unfortunately, infants are one of the highest risk groups for contracting serious infections due to capsule-producing bacteria (e.g., pneumonia and meningitis).

A strategy for improving the immune response to T-cell-independent antigens and extending this response to infants is to covalently link epitopes of the polysaccharide capsule to a protein. This type of vaccine is called a **conjugate vaccine** (more about this in chapter 17). APCs process conjugate vaccines as if they were proteins and elicit a Th2-cell-dependent response that culminates in production of antibodies that also recognize the polysaccharide antigens. This immune response is long-lived because it involves T cell activation and memory cell generation and produces opsonizing IgG1 antibodies, IgG3, and IgG4.

Mucosal Immunity: IgA/sIgA Antibodies

An important immune defense against infectious diseases, but one that is much less well understood than the humoral or cell-mediated responses, is the immune system that produces **sIgA**. The first step in many microbial infections is colonization and invasion of a mucosal surface. sIgA can prevent such infections by blocking colonization. Thus, while the cell-mediated or humoral antibody responses may cause collateral damage to tissues in the area where infection is occurring, the sIgA-mediated defense is usually innocuous to the host because it occurs in the mucus layer.

As we mentioned in chapter 2, skin and mucosal surfaces all have attendant mucosa-associated lymphoid tissues **(MALT)** located just below the epithelial layer in the **lamina propria**. The mucosal surface of the small intestine is underlain with the **GALT**, while the lungs have **BALT,** the upper respiratory tract has **NALT**, and the vaginal tract has **vaginal-associated lymphoid tissue (VALT)**. Skin also has a similar system (called **SALT**).

The intestinal cells that comprise the GALT are visible as a collection of **follicles**, called **Peyer's patches**, which are most highly concentrated in the ileum and rectum of the intestine. Similar mucosal lymphoid tissues are found in the respiratory and vaginal tracts, although they are not as pronounced. The cells that form the Peyer's patches are illustrated in Figure 4-12. **M (microfold) cells** take up antigens from the lumen of the intestinal tract and pass them to closely associated GALT macrophages, which act as the APCs of the GALT. M cells have never been successfully cultivated *in vitro*, so little is known about the activities of M cells. The mechanism by which GALT macrophages process antigens and elicit production of cytotoxic T cells or antibodies is the same as that described in earlier sections, except that the macrophages, B cells, and T cells of the GALT reside specifically in the lamina propria of mucosal surfaces. Two additional types of T cells, Th17 and Tregs, appear to be important for IgA production in the intestine, both of which appear to help modulate the B cells.

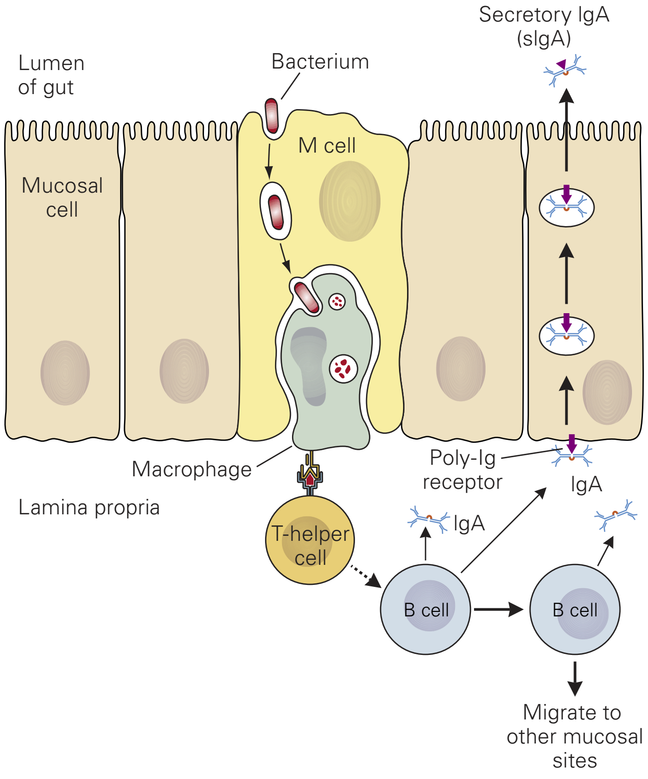

Figure 4-12. Cells of the GALT that confer mucosal immunity. M cells and their associated macrophages and lymphoid cells (T and B cells) are sometimes called follicles. Collections of such follicles in the gut are called Peyer's patches. M cells sample the contents of the gut lumen and transfer the antigens to closely associated resident macrophages, which in turn ingest the bacteria and present antigens to the underlying T cells that then stimulate nearby B cells to produce IgA. The IgA binds to receptors on the basal surface of the mucosal epithelial cell and is transcytosed across the cell and secreted into the lumen of the gut as sIgA.

When the GALT is stimulated, one outcome is production of IgA (see Figure 4-12). Dimeric IgA is produced by plasma cells in the lamina propria at mucosal sites. The secretory piece is acquired when IgA dimer is transported through the mucosal epithelial cell into mucosal secretions covering various mucosal surfaces. IgA dimer binds to the **poly-Ig receptor** on the basal surface of mucosal cells and is then taken up by endocytosis and transported in vesicles, through a process called **transcytosis,** to the apical surface, where it is released into the lumen of the gut. Release involves proteolytic cleavage of the poly-Ig receptor, where the secretory piece comes from the portion of the receptor that remains attached to the IgA, making it sIgA. sIgA can trap microbes in mucus because the Fc portion of sIgA binds to glycoprotein constituents of the mucin and the Fab portion binds to antigens on the microbes in the gut. By trapping the microbes in the mucus layer, the sIgA-antigen-mucin complex essentially forms a protective barrier that blocks the microbes and their toxic products from gaining access to the epithelial cell layer. Mucus laden with sIgA-coated microbes is then sloughed off and excreted from the body.

The mucosal and skin tissues that have contact with the external environment are also constantly patrolled by DCs (APCs), which engulf and kill the invading microbes and process the antigens for presentation to the adaptive immune system. Once activated, DCs migrate to the lymph nodes, where they present the antigens to Th cells and stimulate adaptive immunity. The **Langerhans cells** of the epidermis are the DCs of SALT.

As part of the mucosal immune system, some T cells and B cells stimulated by antigen processing at the GALT can migrate to other mucosal sites and vice versa. Thus, stimulation at one of the MALT sites can transfer to other sites, resulting in general mucosal immunity. The first evidence for this feature of the mucosal immune system came from elegant experiments performed by Husband and Gowans in the late 1970s. These researchers excised a segment of the small intestine from a rat, preserving its vascular and lymphatic supplies, and reconnected the ends of the intestinal segment to the skin surface of the animal, forming a so-called **Thiry-Vella loop** (Figure 4-13). They then introduced an antigen, in this case **cholera toxin (CT)**, into the loop and found that sIgA was secreted in not only the immunized loop segment, but also in a second such loop (when made) and in the main intestine. Their results demonstrated that introduction of an immunogen at one site

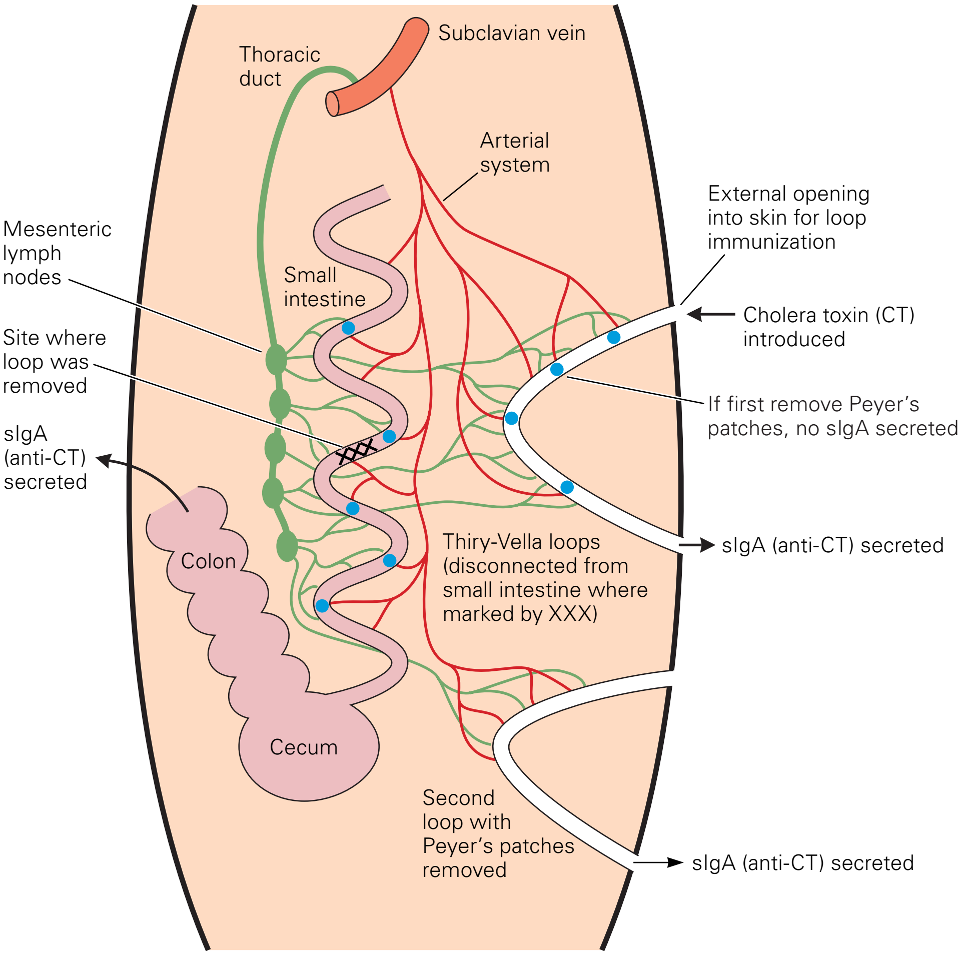
Subclavian vein
Thoracic duct
Arterial system
External opening into skin for loop immunization
Mesenteric lymph nodes
Small intestine
Cholera toxin (CT) introduced
Site where loop was removed
If first remove Peyer's patches, no sIgA secreted
sIgA (anti-CT) secreted
Colon
sIgA (anti-CT) secreted
Thiry-Vella loops (disconnected from small intestine where marked by XXX)
Cecum
Second loop with Peyer's patches removed
sIgA (anti-CT) secreted

Figure 4-13. Classic experiment by Husband and Gowans demonstrating mucosal immunity at remote sites. The experimental setup involved isolating Thiry-Vella loops of the small intestine of rats and connecting them to the skin, preserving the associated vascular and lymphatic systems attached to the loops. The IgA immune response to administering immunogen (cholera toxin) to the main intestine through the oral route or through the skin opening of the loop could then be observed. Results showed that local immunization through the isolated loops that contained Peyer's patches generated Th cells and B cells that circulated through the lymph and blood to populate other mucosal sites. Based on Husband AJ, Gowans JL. 1978. *J Exp Med* **148:**1146–1160.

could confer mucosal immunity at a remote site. This characteristic of the MALT system is what makes **oral vaccines** feasible. Initially, oral vaccines stimulate the GALT, but sIgA against vaccine antigens is later detectable in other MALT sites. Thus, an oral vaccine can also be used to elicit immunity to respiratory and, presumably, urogenital pathogens.

Currently, efforts are being made to develop vaccines administered by inhalation, so that stimulation of the nasal MALT (NALT) would produce an sIgA response at other MALT sites. These vaccines would have the advantage of not having to pass through the stomach. Developing vaccines that target the GALT means developing vaccines capable of surviving the low-pH/protease-rich stomach environment, a barrier that has proven problematic in many cases. Administering vaccines by rectal or vaginal suppositories is theoretically possible, but this strategy has not been actively pursued to date. On the other hand, the SALT is gaining in attraction as a target for vaccine development (more on vaccines in chapter 17).

Activation of the GALT can also lead to production of cytotoxic T cells. These cells probably remain on the basal side of the mucosa, although it is possible that during an infection some of them migrate to the apical surface, especially in areas where damage to the mucosa has occurred. GALT cytotoxic T cells are important for protection against viral infections of the GI tract and some bacterial infections in which the bacteria multiply inside mucosal cells.

One of the many mysteries swirling around the intestinal immune system is the role of a particular type of mucosal cell called γδ **T cells.** The majority of γδ T cells are CD8$^+$ T cells and would thus be grouped with CTLs. However, whereas CTLs have TCRs composed of αβ chains (Figure 4-8), the **intestinal epithelial lymphocytes (IELs)** have T-cell receptors composed of related but somewhat different γδ chains.

These γδ T cells account for less than 4% of circulating CD8$^+$ T cells, but they account for as much as 10 to 15% of the mucosal T cells found in the GI tract. In some regions, such as the colon, the levels may be as high as 40%.

Unlike αβ T cells, γδ T cells seem to recognize only a limited number of cell-surface antigens. Also, γδ T cells seem to bypass antigen presentation by MHCI and MHCII on the surfaces of macrophages and DCs and directly recognize nonpeptide antigens that have not been processed. Indeed, they are believed to play a predominant role in lipid-antigen recognition via CD1 complexes (Figure 4-6C). γδ T cells also respond to two human protein complexes related to MHC I, **MICA** and **MICB**. These proteins are displayed on the surface of cells that are stressed (e.g., by being infected) and are recognized by NKG2D receptors present on γδ T cells, as well as NK cells, which results in activation of cytotoxic responses that kill the stressed cells. γδ T cells also produce cytokines that stimulate αβ CD8$^+$ cytotoxic T cells to migrate to the area and eliminate infected cells.

As with other immune responses, the GALT has a downside. Normally, bacteria and other viable antigens that pass through M cells are killed by the resident GALT macrophages. Some bacterial pathogens, however, have acquired the ability to avoid this fate and exploit the GALT as an entryway into the body. Because the M cell is an antigen-sampling cell, it usually does not take up substantial amounts of an antigen because only a few bacteria or other antigens are sufficient to stimulate a mucosal immune response. The bacteria that use the GALT as an entryway into the body, such as *Salmonella enterica* or *Yersinia pseudotuberculosis*, invade M cells or use them to move across the epithelial layer (see Figure 4-14).

Development of the Adaptive Immune System from Infancy to Adulthood

Human infants do not develop fully effective immune defenses until they are one to two years of age. Infants have very low levels of NK cells and are deficient in mannose-binding proteins, one of the acute-phase proteins produced by the liver that helps opsonize some types of microbes. For the period up to one year of age, maternal antibodies that were transferred through the placenta during gestation or ingested in milk during infancy protect the infants at least partially. The infant's antibody production begins to kick in at about two to three months of age, but the rate of production is still relatively slow with antibodies being short-lived and of low avidity. T-cell-independent responses do not start until about 18 months of age. The transition between the protection conferred by maternal antibodies and the development of the infant's own immune system provides pathogenic microbes with a window of opportunity and is one of the reasons why children under the age of two are particularly vulnerable to infectious diseases.

Interestingly, circulating maternal antibodies can actually interfere with an immune response to some antigens, because high levels of antibody to a particular epitope discourage the development of an antibody response to that epitope. This is a consequence of immune regulation that normally protects the body from overreacting to a particular epitope. The dampening effect of circulating maternal IgG is the reason why some vaccines are not given to infants less than one year of age.

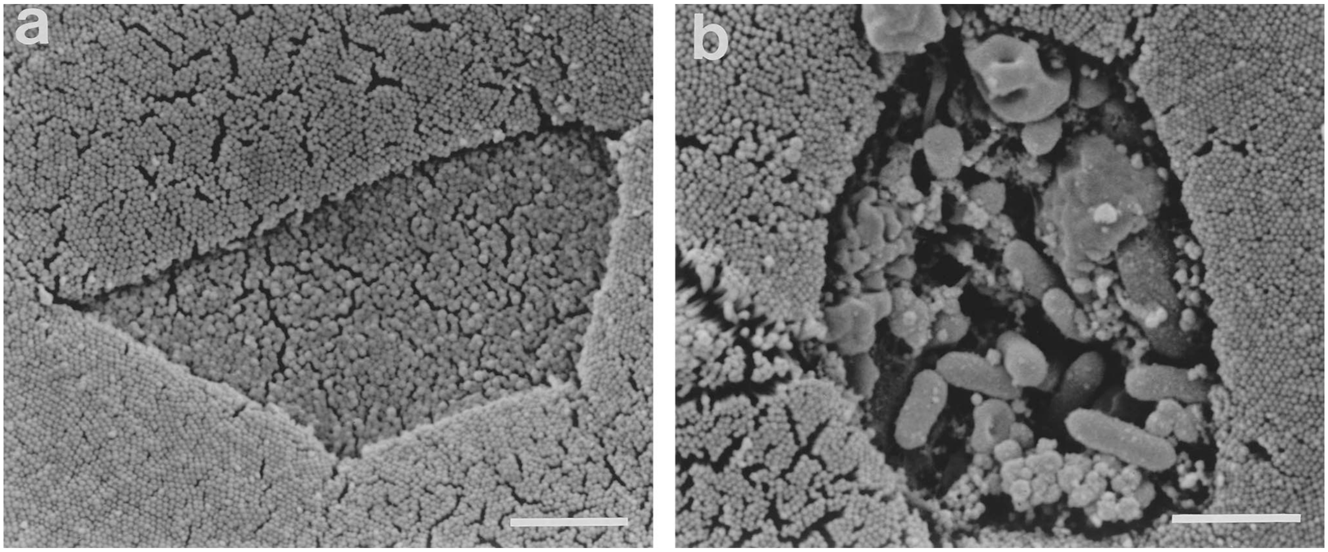

Figure 4-14. Scanning electron micrographs of mouse Peyer's patches (a) before and (b) after incubation with *Yersinia pseudotuberculosis*. Both images depict a central M cell surrounded by enterocytes (and part of a brush cell in the lower left of panel b). The M cell in panel a lacks adherent bacteria but, in contrast, the association of bacteria with the M cell in panel b is accompanied by disruption of the normal surface morphology of the M cell. Bars, 2 μm. Reprinted with permission from Clark MA, Hirst BH, Jepson MA. 1998. *Infect Immun* **66**:1237–1243.

Adaptive Defense Systems in Nonmammals

Scientists have found primitive versions of the mammalian adaptive defense system in many different organisms, ranging from insects to sharks (Table 4-2). Insects such as *Drosophila melanogaster* (fruit fly) and slime molds such as *Dictyostelium* also have primitive defense systems that resemble mammalian defense responses, especially the innate immune response. Zebra fish not only have an innate immune response but also have a response that parallels the Th1/Th2 adaptive response. Sharks produce antibodies that can bind to human pathogens.

Even plants have a primitive defense system in which proteins produced by the plant (R proteins) bind to invading bacteria, a reaction that produces apoptosis in the surrounding plant cells. This area of dead, dry tissue, which appears as black spots on leaves or fruit, traps the bacteria in the area. Bacteria need to move throughout the plant via the xylem and phloem in order to cause serious disease, and the plant's defense response is designed to prevent this.

Assertions that systems in nonmammals are "immune systems" should be viewed with some skepticism. In the case of sharks, plants, and zebra fish, there is evidence that the systems are actually a response that protects the organism from infection. In the case of *Drosophila,* however, the Toll receptors that led to the discovery of mammalian Toll-like receptors, which are a central part of the mammalian innate immune response, appear to be involved primarily in development of the insect or in feeding behavior. Only one Toll receptor appears to play a role in defending the insect against fungal infections. Nonetheless, it is intriguing to see that parallels of what we know as the mammalian innate and adaptive defense systems are so widespread in other creatures. Interest in the evolution of the mammalian immune system is a relatively new area of research focus, so there are likely to be many more insights in the future as this research progresses.

The Dark Side of the Adaptive Defenses: Autoimmune Disease

Complex figures such as those shown in this chapter illustrate the manifold interactions that occur between cells of the immune system when they activate each other. All of those proteins labeled CD followed by a number have an important role. They help ascertain that the contacts between the immune cells are occurring in response to true foreign antigen presentation events and not accidental associations between cells of the immune system and other cells in the body. They serve as insurance that the immune system will not go out of control and attack self cells.

Unfortunately, this safeguard is sometimes breached, like when bacterial antigens contain epitopes that mimic human antigens. In such cases, the adaptive defenses can produce antibodies or CTLs that mount an attack against the host, thereby creating a set of conditions that can lead to **autoimmune disease**. If the targeted tissue is the heart or another vital organ, the misguided attack can be lethal. In later chapters, we will encounter examples of autoimmunity initiated by the adaptive immune defenses against bacterial components that mimic host antigens. Bacteria that elicit an autoimmune response are particularly difficult targets for vaccine development. Such a vaccine could induce an autoimmune response when the vaccinated person is exposed to the bacterium, which could make the disease worse rather than preventing it. Thus, one of the concerns of vaccine producers is to make sure that their vaccine does not elicit an autoimmune response.

Table 4-2. Defense systems in higher organisms

ORGANISM	FORM OF INNATE IMMUNITY	FORM OF ADAPTIVE IMMUNITY	INVASION-INDUCED SIGNALING	PHAGOCYTIC CELLS	ANTIMICROBIAL PEPTIDES	PATTERN RECOGNITION RECEPTORS	T AND B CELLS	ANTIBODIES
Plants	+	−	+	−	+	+	−	−
Invertebrates								
Sponges	+	−	?	+	?	?	−	−
Worms	+	−	?	+	?	?	−	−
Insects and crustaceans	+	−	+	+	+	+	−	−
Vertebrates								
Sharks	+	+	+	+	+	+	+	+
Bony fish	+	+	+	+	+	+	+	+
Amphibians	+	+	+	+	+	+	+	+
Reptiles	+	+	+	+	?	+	+	+
Birds	+	+	+	+	+	+	+	+
Mammals	+	+	+	+	+	+	+	+

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Questions

1. In the short statement that began the chapter, the innate immune system was likened to ordinary police and the adaptive immune system was likened to specialized backups such as snipers. For those of you who follow true crime or mystery stories, you might find it amusing—and helpful in your studies—to try to come up with other criminal justice analogies. For example, you might make an analogy between "antibody production by B cells" and "smart bombs, surveillance, and tracking devices." See what you can do with T helper cells, cytokines, APCs, and NK cells.

2. Does the fact that sIgA does not activate complement make it less effective in preventing infection than IgG? Hint: Consider its location and function.

3. Explain how the structure of an antibody is designed to facilitate the function of the particular antibody (e.g., why an antibody has at least two active sites, why it has an Fc portion, and why it might be cross-linked as a multimer).

4. Why is a Th1 or Th2 cell needed to mediate APC-initiated antibody production? Why do the B cells not bind directly to the APCs?

5. Explain why direct antigen activation of cytotoxic T cells and B cells is more important than APC-initiated activation in subsequent encounters with a microbe. Why is the APC-initiated pathway needed at all?

6. There are many interactions between the innate and adaptive defense systems. List as many of them as you can and explain how they work. Why are these interactions important? Are all of them beneficial?

7. What are the two ways that polysaccharides, DNA, and lipid antigens can elicit an immune response? How are these responses different than that elicited by a peptide antigen?

8. Why is the level of specific IgM antibodies of diagnostic significance?

Solving Problems in Bacterial Pathogenesis

1. A group of researchers have isolated a new pathogenic bacterium Q from lungs and lymph nodes of cystic fibrosis patients that produces an unusual polysaccharide capsule, called QPS. They can see QPS on the surface of the bacteria in electron micrographs from fresh isolates (i.e., bacteria just obtained from patients). In addition to the capsule, bacterium Q produces a protease, called QP, which the researchers believe is responsible for degrading host sIgA.
   a. The researchers propose that QPS and QP might make good targets for vaccine development against bacterium Q. What led the researchers to propose this?
   b. What potential problems could the researchers have in using QPS as a vaccine?
   c. How are QP antigens presented to immune cells?
   d. The researchers find that the QPS vaccine does not elicit a long-lasting immune response. How does QPS elicit an immune response? What vaccine strategy could be used to generate long-lasting immunity targeting QPS?
   e. The researchers believe that they can develop an oral vaccine that will be effective against lung infections with bacterium Q using QP as a vaccine component. What specific experimental evidence supports their rationale for using this proposed approach?
   f. If QP were used as a vaccine, what immune response would be primarily responsible for controlling lung infection caused by bacterium Q?

2. *Listeria monocytogenes* is a foodborne, Gram-positive bacterium that causes infections (listeriosis) in individuals with compromised immune systems. *Listeria* is predominantly an intracellular pathogen. After phagocytosis by intestinal macrophages, *Listeria* escapes the phagosome and replicates in the cytosol before spreading to other neighboring cells through a special invasion process that allows it to remain intracellular while spreading from cell to cell.
   a. What advantage is there for *Listeria* to invade and spread intracellularly?
   b. What immune response is primarily responsible for controlling *Listeria* infection in healthy but unimmunized individuals?
   c. What immune response is primarily responsible for controlling *Listeria* infection in healthy but immunized individuals or those having prior exposure?
   d. How are *Listeria* antigens presented to immune cells?

3. Lyme disease is a common zoonotic vector-borne disease in the United States. It is caused by the Gram-negative spirochete *Borrelia burgdorferi*, which is transmitted through the bite of infected deer ticks. After a period ranging from 3 to 30 days, a unique red rash with a classic bull's-eye appearance occurs in ~75% of infected persons at the site of the bite (shown in Figure 1). Though such a rash does not always appear, the presence of this characteristic rash is diagnostic for Lyme disease (there are no other known causes for it), so physicians often prescribe antibiotics without further diagnostic testing. In most cases, antibiotics such as tetracycline and penicillin eliminate the infection and its symptoms. However, delayed or inadequate treatment can lead to more serious symptoms much later, including effects on the joints (chronic arthritis, inflammation), the heart (atrioventricular blockage, abnormal heart rate), and the central nervous system leading to facial palsy (paralysis), meningitis (headaches, neck stiffness), or encephalitis (memory loss, mood changes). Upon injection of the bacteria into the skin by the tick, the bacteria multiply and migrate slowly outward within the dermis. Without antibiotic treatment, the bacteria eventually spread throughout the body, despite generation of high titers of antispirochete antibodies. Microscopic examination of various tissues during infection reveals that these spirochetes are not found inside host cells, but instead reside within the extracellular matrix of skin, joints, and heart, peripheral, and central nervous systems.

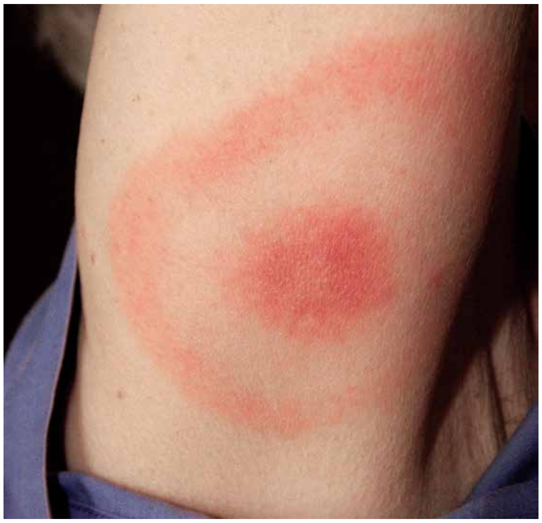

Figure 1. The characteristic bull's-eye rash (erythema migrans) caused by Lyme disease. Image from CDC-PHIL ID#9875, courtesy of CDC/James Gathany.

a. What is the cause of the characteristic bull's-eye rash that appears around the tick bite without antibiotic treatment? Be sure to provide a plausible explanation for the bull's-eye appearance.
b. Which cells would normally be the first on the scene to eliminate the spirochetes from the bite site? Be specific.
c. Interpret what it means to have the occurrence of strong antibody responses against *B. burgdorferi* in chronically infected individuals in the absence of sterilizing immunity.
d. Provide two possible explanations with rationale for why these cells might not respond effectively to clear the spirochete infection. For each of these possibilities, provide at least one definitive experiment that could be performed to confirm the explanation.
e. Provide a possible explanation with rationale for the later symptoms of inflammation and arthritis associated with chronic infections.

5

IN THIS CHAPTER

Importance of the Normal Resident Microbial Populations (Microbiota) of the Human Body

Characterization of the Body's Microbiota

- Taking a Microbial Census by Using Microbial rRNA Gene Sequence Analysis
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CHAPTER 5

The Microbiota of the Human Body

MICROBIOMES AND BEYOND

The ancient Greek philosopher Protagoras of Abdera (ca. 490–420 BCE) famously declared that "Of all things, the measure is Man," but to the microbes that live in or on us we are more like the proverbial "free lunch." Yet the microbes colonizing our bodies from shortly after our birth to our death do "pay rent" in various ways. They protect us from disease-causing microbes and contribute to our nutrition and healthy immune status. Our bodies are adapted not only to tolerate these resident microbes, but also to encourage their presence. Indeed, getting in touch with our microbial side is an important part of understanding what it is to be human.

Importance of the Normal Resident Microbial Populations (Microbiota) of the Human Body

A healthy human body harbors more than 10 times as many microbial cells as human cells. These microbes, which include bacteria, fungi, and archaea, are collectively known as the **microbiota.** Since they do not take up as much space as human cells, their bulk is less than that of the human cells, but their various manifestations can be just as noticeable. For example, the odor of sweat comes from bacterial metabolism of compounds in skin secretions. Bacteria form the film that develops on unbrushed teeth and the plaque that we pay dental hygienists to remove. Even the flatus that we embarrassingly emit is an odiferous indication of the activities of bacteria in the intestine.

Although these particular manifestations of the presence of bacteria are viewed by most as unpleasant, eliminating bacteria from our bodies, even if it were possible, would be a very bad idea. These tiny freeloaders play many beneficial roles in our health. Many members of our resident microbiotas provide us nutrients by degrading foodstuffs that remain undigested by our own systems, such as complex carbohydrates, and by synthesizing and excreting essential vitamins, such as vitamin K, vitamin B12, and other B vitamins.

Normal microbiota also protect us from pathogens by competing for nutrients and occupying potential attachment sites. Indeed, the importance of these resident microbiota becomes particularly evident when they are disrupted or eliminated. For example, a common symptom of taking antibiotics is the development of mild diarrhea as the composition of the microbiota of the colon is disturbed. In more extreme cases, toxin-producing bacteria, such as *Clostridium difficile*, that are normally present in very low numbers can grow out of control, leading to severe damage of the colon lining and even death. Women who have taken antibiotics that affect the balance of the normal vaginal microbiota often develop vaginitis caused by overgrowth of yeast or vaginosis caused by overgrowth of certain Gram-negative bacteria. Under normal conditions, the vagina is colonized predominantly by Gram-positive *Lactobacillus* species. When the numbers of these protective *Lactobacilli* decrease, yeasts and other Gram-negative bacteria can overgrow and produce toxic substances or degradative enzymes that further kill members of the normal microbiota, cause damage to the host mucosa, and ultimately cause disease symptoms, such as foul odor or discharge.

Recent experimental evidence indicates that indigenous bacteria play a crucial inducive role in intestinal development during early postnatal life, particularly in the caecum and Peyer's patches, as well as in development and maturation of the innate and adaptive immune systems. Colonization by commensal microbiota stimulates the production of cross-reactive antibodies, which are secreted into the lumen of the gut where they prevent infection by related pathogens. Experimental evidence has demonstrated that animals that are born in aseptic conditions and raised in a sterile environment without exposure to microorganisms (**germfree animals**) have severely underdeveloped immune systems and require substantially more food to maintain the same body weight as others that are raised conventionally.

The notion that maintaining beneficial or "good" bacteria in the intestinal tract is conducive to good health has actually spawned the billion-dollar **probiotics** industry. Probiotics are preparations of live bacteria, usually freeze-dried into pellets or added to foodstuffs such as yogurt, that are ingested intentionally to bolster the normal population of "good" colonic bacteria or are included in douches to promote a healthy vaginal microbiota. Most of the preparations contain *Lactobacillus* or *Bifidobacterium* genera that are common to a particular body site. While the particular strains commonly used in commercial preparations are considered safe, they often do not colonize the host well and so must be taken frequently to maintain the bacteria in the host environment. An alternative approach to maintaining a healthy microbiota that is gaining popularity is **prebiotics**, which are substances such as fructo-oligosaccharides or nondigestible fiber or "roughage" derived from plants that can alter the composition of the gut microbiota by fostering the growth of "good" bacteria in the large intestine.

Claims for the probiotics currently on the market tend to be vague and very general, but credible scientific evidence is mounting that these preparations may provide some long-lasting health benefits. The idea that probiotics or prebiotics could help patients whose intestinal or vaginal bacterial populations have been disrupted by antibiotics or other factors has attracted new interest for biomedical applications. The hunt for new, targeted therapies that restore optimal balance within microbial communities has already yielded alternative clinical treatment strategies, such as strain supplementation mentioned earlier and prophylactic fecal transplantation to prevent disease from overgrowth of *C. difficile*. New information on the composition and properties of the normal microbiota of our bodies may further improve the design of effective probiotics.

In a related area, researchers are beginning to exploit our microbiota as a powerful diagnostic tool for assessing health status. For instance, accurate diagnosis of changes in the microbiota might help identify patients who are at higher risk for certain diseases, particularly those diseases whose manifestation correlates with a perturbed microbiota. Ideally, intervention strategies could be initiated sooner and with greater certainty of outcome in these individuals. Understanding the role of our microbiota in health and disease is already having a tremendous impact in the area of **precision medicine**, an emerging personalized and holistic medicinal approach to disease prevention and treatment that takes into account environmental, lifestyle, life history, and genetic variability of each individual.

Figuring out how to maintain bacterial populations that are conducive to health and then finding ways to capitalize on that knowledge to promote a healthy microbiota is not a trivial undertaking. The microbial populations found in the mouth, intestine, and vagina are complex, not only in terms of the hundreds of different species at each site, but also in terms of the diversity of the metabolic requirements and outputs of those species. Until recently the only way to characterize the microbiota of a particular body site was microscopic visualization and/or laboratory cultivation using various growth media. This approach was not only time-consuming and expensive, but also unreliable because of the poor plating and growth efficiencies of the organisms that are more difficult to cultivate, which often comprise the majority of the bacteria. Additionally, many bacterial species cannot even be cultured in the laboratory. As a result of these challenges, microscopy or culture-based approaches are simply unable to address important questions regarding microbiota makeup, spatial and temporal dynamics, and contribution to health and disease. In order to tackle these unknowns, new technologies and approaches were required.

Characterization of the Body's Microbiota

The advent of culture-independent, nucleic acid-based methods for characterizing bacterial populations revolutionized how researchers acquired information about the composition and diversity of bacterial communities and revitalized research interest in the mammalian microbiota. A critical advance over the past decade that changed this picture even more was the development of mega-scale, high-throughput, rapid, and cost-effective DNA sequencing technologies and the accompanying analytical and bioinformatics tools needed to manipulate, interpret, integrate, and store these large data sets. Importantly, the dramatic reduction in sequencing and computational costs in the last few years has moved the field forward at an astounding pace. Not only is it possible to quickly sequence genomes from isolated, individual microbes (**genomics**), but now the entire assemblage of genomes within complex microbial communities (**microbiomes**) can also be determined with relative ease.

Parallel to these remarkable advances in DNA sequencing and analysis, other momentous advances have occurred: in RNA sequencing and gene expression technologies (**transcriptomics**) for the assessment of which genes are expressed under particular conditions; large-scale separation, identification, and analytical tools for assessment of protein expression and function (**proteomics**); multiplex antibody, cytokine, and chemokine immunologic profiling for assessment of the host immune response; and lipid, sugar, and other metabolite profiling (**metabolomics**) for the assessment of cellular processes of individual microbial cells as well as those of the entire microbial community and the host. Indeed, many researchers are now combining these various technologies into multivariate holistic approaches (**multiomics**) that enable overall functional assessment of the microbiota in the context of the host environment.

Before delving into the microbial populations of different parts of the body, it is worth reviewing some of the powerful new culture-independent approaches and analytical tools that are available for exploring the scope, depth, and variety of microbes that comprise mammalian microbiotas.

Taking a Microbial Census by Using Microbial rRNA Gene Sequence Analysis

Some of the first questions that arise when scientists are seeking to characterize a complex microbial population are: what microbial species and how many of each are present, how much variation in composition is there from person to person and site to site, and how does the composition change with conditions and over time? To answer these questions, it is first necessary to identify the species to which the microbes belong and to determine their phylogenetic relationship (or evolutionary similarity) to each other. Although most studies surveying the microbiota to date have focused on the bacterial content of the community, there are some researchers who are beginning to explore other microbes, such as archaea, fungi, protozoa, and viruses (including bacteriophage). For now, we will likewise focus on characterization of the bacterial content of microbiotas, but will return to the other microbes later in the chapter.

16S rRNA Gene-Based Taxonomical Identification of Bacteria. To complete a census of the species present in a bacterial community, researchers must first perform sequence analysis of all, or at least the more abundant, species present in a sample. For this, they need to choose a gene that is common to all bacteria of interest. The most widely used approach to determining the bacterial content of a community is to isolate the total genomic DNA from the microbial population and then employ **polymerase chain reaction (PCR)** to specifically amplify the bacterial 16S rRNA genes (Figure 5-1), which are then sequenced. The use of 16S rRNA genes is advantageous because these genes are large enough (1,542 nucleotides for the *Escherichia coli* gene) to contain adequate sequence information for identification and discrimination among close relatives but small enough to be sequenced easily. The 16S rRNA gene, which is present in all bacteria, is a mosaic of regions (Figure 5-1A), some that are highly conserved among all bacterial species and some that are less conserved and consequently contain sequence signatures for different bacterial species, acquired through slow accumulation of mutations over time.

The basic procedure is relatively simple. DNA primers that recognize highly conserved regions at the beginnings and ends of the bacterial 16S rRNA genes are used to amplify most of the gene, including the variable regions containing the identification signatures, by PCR using a thermostable, high-fidelity DNA polymerase, such as Vent or Pfu (Figure 5-1B). The resulting PCR products, called **amplicons**, are sequenced directly using new DNA sequencing technologies, described in detail later in the chapter. Using **bioinformatics** (computer software programs capable of handling and analyzing massive amounts of data), the sequences of the rRNA genes present in the original sample (called output sequence reads) are compared to those available in the ever-growing, publicly available DNA sequence databases (Box 5-1) to identify the nearest bacterial relatives and provide an immediate identification of the **taxon** (plural **taxa**; group of one or more populations of related organisms) from which the sequence originated. It is now possible to identify an unknown bacterial isolate within 24 hours by this approach, and with automation, high-capacity supercomputers, and current bioinformatics tools, it can happen even sooner.

The realization that sequencing the 16S rRNA genes can be used to rapidly and accurately identify bacterial strains introduced a new era in bacterial detection and identification. Because the primers recognize conserved regions of the 16S rRNA gene, which are universal in bacteria, this approach can be used to rapidly identify bacteria that are not amenable to cultivation. The revolution first hit in environmental microbiology, for which nothing equivalent to the detailed identification protocols of clinical microbiology existed and the vast majority of the bacteria could not be cultured using conventional media. One of the first successes of this approach in clinical microbiology was the identification of the bacterium that causes a rare form of intestinal disease called Whipple's disease. A bacterium-like form could be seen in tissues of infected people but attempts at cultivation had been unsuccessful. Finally, using this technique, the Gram-positive bacterium associated with Whipple's disease was identified as *Tropheryma whipplei*.

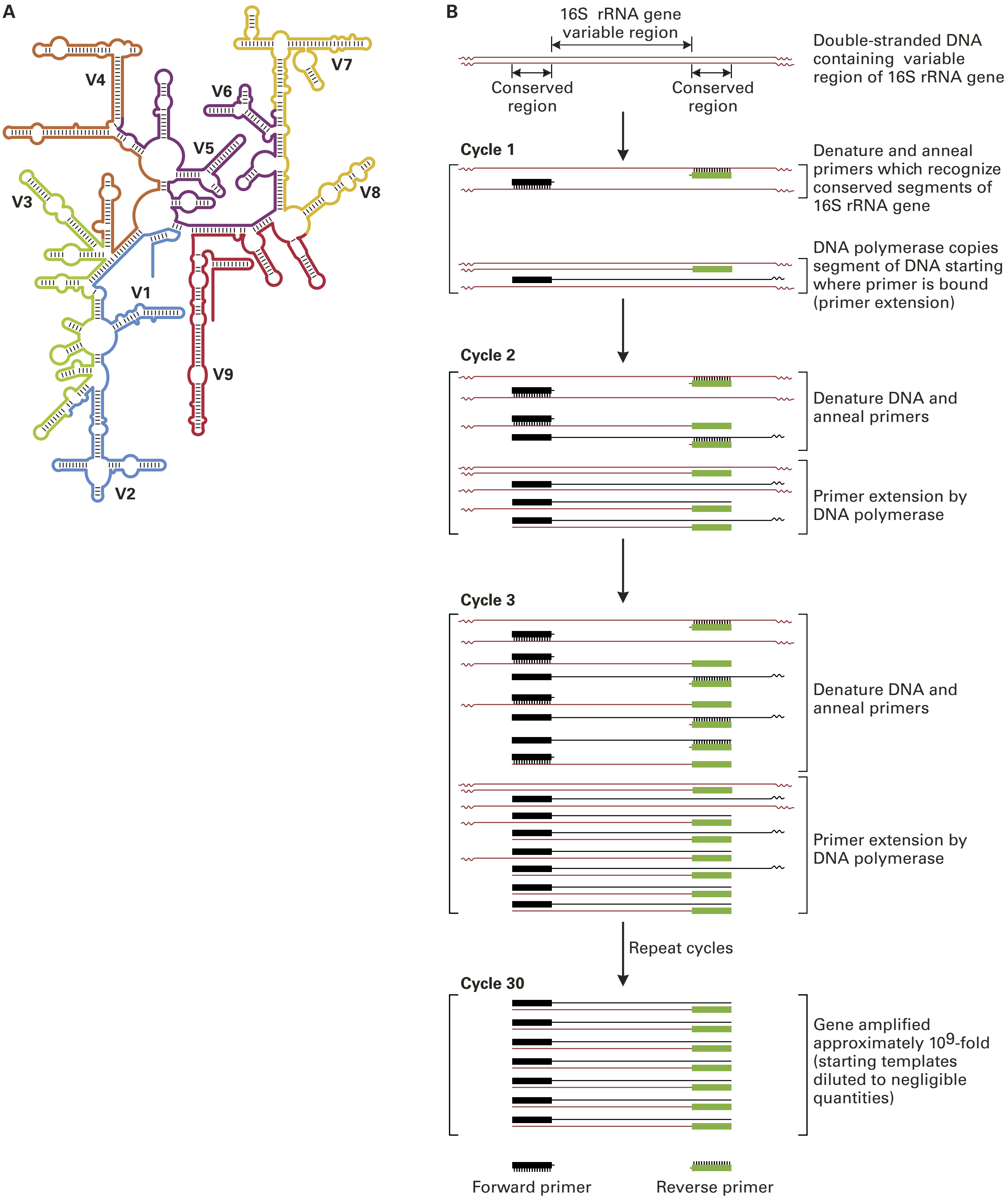
A
V4
V6
V7
V5
V3
V8
V1
V9
V2
B
16S rRNA gene variable region
Conserved region
Conserved region
Double-stranded DNA containing variable region of 16S rRNA gene
Cycle 1
Denature and anneal primers which recognize conserved segments of 16S rRNA gene
DNA polymerase copies segment of DNA starting where primer is bound (primer extension)
Cycle 2
Denature DNA and anneal primers
Primer extension by DNA polymerase
Cycle 3
Denature DNA and anneal primers
Primer extension by DNA polymerase
Repeat cycles
Cycle 30
Gene amplified approximately 10⁹-fold (starting templates diluted to negligible quantities)
Forward primer
Reverse primer

Figure 5-1. Detection of bacteria in a clinical specimen based on 16S rRNA gene amplification by PCR. The 16S rRNA gene is used as the standard for bacterial taxonomic identification and phylogenetic relationship studies because it is highly conserved among different taxa of bacteria. **(A)** The 16S rRNA gene (~1,542 nucleotides in the *Escherichia coli* gene shown here) has highly conserved regions of sequence that can serve as primer binding sites for PCR amplification, but also has hypervariable regions (labeled V1 through V9) that can be used as signatures for distinguishing among different bacterial taxa and establishing phylogenetic relationships. **(B)** For detection of bacteria in a clinical sample, PCR primers (solid dark bars) recognize conserved segments of DNA on either side of the variable region to be amplified. For the PCR reaction, a thermostable DNA polymerase that exhibits maximal catalytic activity at 75–80°C and possesses 3′ to 5′ exonuclease activity that reduces incorporation of the wrong nucleotide is used, such as the Pfu or Vent polymerase. The amplified segment (amplicon) can then be sequenced and compared to rRNA databases of known bacteria for taxonomic identification.

Box 5-1.

Data, Data, Data—What To Do with All That Data?

How does one go about storing and sorting through the massive amounts of sequencing data and information that has been generated over the years? Because of the critical need for researchers to have access to the data and be able to readily use it, a number of centralized, publicly available databases have been formed around the world. These **databases**, most of which are Web-based and freely available online to the public, consist of libraries of life sciences information, DNA sequencing data, protein structure data, gene expression data, and other computational or scientific data from genomics, transcriptomics, proteomics, metabolomics, and phylogenetics. Because of the need for compiling and analyzing these massive amounts of data and information from various sources, an entirely new field of **bioinformatics** emerged that involves design, development, management, utilization, and maintenance of these life sciences databases. Databases have become an important tool and resource for scientists studying complex biological systems. Whenever a researcher obtains or publishes a nucleotide sequence or other data in a scientific journal, the researcher is required to deposit that sequence and/or information in one of the databases, and that sequence receives an **accession number**, which is a tracking number that helps the databases maintain and cross-reference the information.

The largest primary sequence databases, which form part of the **International Nucleotide Sequence Database** (INSD), consist of: **GenBank** (National Center for Biotechnology Information (**NCBI**)), the United States' centralized library of various biological data, including nucleotide sequences; **EMBL ENA** (European Molecular Biology Laboratory European Nucleotide Archive), Europe's library of nucleotide sequence data; **DDBJ** (DNA Data Bank of Japan), Japan's nucleotide database; **UniProtKB** (Universal Protein Resource Knowledgebase), a database that provides protein translations of nucleotide sequences from the nucleotide sequence databases; **Swiss-Prot** (Swiss Institute of Bioinformatics), a protein sequence database; and **RCSB PDB** (Research Collaboratory for Structural Bioinformatics Protein Data Bank), a protein structure model database.

There are public genome databases that collect libraries of genome sequences and provide annotation (assigning identification and possibly function to the genes), curation (literature citations supporting the annotation), and analysis tools to aid researchers in comparative genomics studies. For example, **JGI Genomes** (Department of Energy Joint Genome Institute) is a database for many eukaryotic and microbial genomes, the **NMPDR** (National Microbial Pathogen Data Resource) is a curated database of annotated genomic data for a number of bacterial pathogens, and the **SEED** is a database developed to annotate and curate 1,000 genomes using a subsystems approach based on comparative analysis of sets of genes with related functional roles. Some databases, such as **KEGG Orthology** (Kyoto Encyclopedia of Genes and Genomes), **COGs** (Clusters of Orthologous Groups), **eggNOG** (evolutionary genealogy of genes: Non-supervised Orthologous Groups), and **Pfam** (Protein Families and Domains), map DNA and RNA sequence reads and functional information into evolutionarily related families of similar biological activities, metabolic pathways, and/or protein structure and function. There are also databases that integrate information from multiple databases. For example, **Entrez** is the integrated search and retrieval system used by NCBI for assembling data from major life sciences databases, including literature sources (such as **PubMed**), nucleotide and protein sequences, protein structure, taxonomy, genome, expression, chemical, and other databases, and it makes the resulting combined information available to the public through a single platform (URL: http://www.ncbi.nlm.nih.gov). Similarly, **UniProt** is a European database that is comprised of four core databases for protein sequence and function.

One of the greatest challenges with having so much data and information available is that it is difficult for the databases to verify the input data. While some database resources try to maintain oversight, it is often left to the researchers who deposit the data to annotate and curate their data. This is not always a reliable way to ensure that the data are correct, so the end user must also be wary and take care not to use false data. Often, what happens is the researchers deposit large quantities of sequencing data, for which no annotation or curation has occurred. This is becoming ever more prevalent with the massive metagenomic sequencing efforts that are currently underway, such that the number of 16S rRNA gene sequences of uncultured microorganisms has already far surpassed the number of cultured microorganisms. Defining taxonomic thresholds for separating cultured bacteria and archaea based on 16S rRNA gene sequence comparison is already challenging in terms of the diversity observed, even when nearly the entire gene is used. Taxonomic classification of uncultured bacteria and archaea, particularly using information obtained from only partial gene sequences (short reads) typically obtained through large-scale metasequencing platforms, is currently one of the most fundamental problems in microbiology.

To deal with this issue of sorting microorganisms into defined taxa at the species, genus, or higher taxonomic ranks using strictly molecular genetic criteria, some databases have the capability of matching sequences from a sequence library obtained from known, well-characterized bacteria or archaea (so-called type strains), which allows one to link taxonomy with phylogeny. One such database is the **Ribosomal Database** (**RDB**, URL: http://rdp.cme.msu.edu), which provides online data analysis, alignment, and annotation of bacterial and archaeal small-subunit 16S rRNA gene sequences and fungal

(*continued*)

Box 5-1.

Data, Data, Data—What To Do with All That Data? (*continued*)

18S rRNA gene sequences from a sequence library of only type strains or from the entire collection of sequences regardless of annotation (type and non-type strains). In addition, the RDB provides alignments for sequence comparisons and phylogenetic analysis that incorporates information from the conserved secondary structure of 16S rRNAs, which enables improved comparisons of short partial sequences and handles some artifacts that might arise from large-scale sequencing.

But the best way to experience the amazing power of bioinformatics is to try it for yourself. Go to the Entrez site or another database and type the name of your favorite protein. You will be amazed at the depth of information that is available about this protein: which species produce it; phylogenic relationships of the protein in different species; its structure (often done by different methods and perhaps even bound to ligands); the possible functions of its domains and how these domains relate to related domains in other proteins; how its expression is regulated at the gene and activity levels; where it fits in metabolism or cellular processes; signal transduction pathways that impinge on it; and on and on. The total amount of new biological information may seem daunting, but you can best appreciate the new depth of the current biological revolution by plunging in and looking for yourself. Besides, the structures and relationships are truly beautiful—and it is all free!

The 16S rRNA gene sequence profile of the microbial community present in a sample can be represented as a **phylogenetic tree**, such as that illustrated in Figure 5-2, which shows the evolutionary relationship of the bacteria to each other based on sequence similarity of the reads. Each branch point (or **node**) is a **taxonomic unit** that represents the most recent common ancestor of the descendants. The lengths of the branches represent estimated sequence similarities or relationship distances from each other, from which estimates of evolutionary time can be inferred. An **operational taxonomic unit (OTU)** is a term used to group closely related organisms based on their sequence similarity of a specific taxonomic gene, such as the 16S rRNA gene. Usually when analyzing microbiotas, the researcher sets a threshold sequence similarity of 97%, 98%, or 99% to define a cluster of OTUs, but this can vary and may be influenced by errors in DNA sequencing.

Another common method for depicting phylogenetic relationships or similarity (in terms of distance from each other) among microbial profiles is to use mathematical ordination methods, which are multivariate algorithms in which species units (taxa) or distance profiles of communities are clustered (or ordered) along gradients. One such method, called **principal component analysis (PCA)**, generates a PCA plot from a microbial community data matrix, in which rows are taxa (or relative abundance or similarity distance) and columns are samples (Figure 5-3A). In a PCA plot, the observed data are mathematically transformed into a coordinate system (x,y-plot or x,y,z-plot), such that the data are projected to fit within the plot, where each axis (coordinate) represents a variable called a principal component (PC). The *x* axis (PC1) represents the component with the greatest variance (i.e., has the factor 1 that accounts for most of the variability in the data) and the orthogonal (perpendicular) *y* axis (PC2) represents the component with the second greatest variance (i.e., has another factor 2 that accounts for the next highest variability in the data). As for a phylogenetic tree, the closer the points are clustered together, the more similar the sample profiles are to each other (Figure 5-3B).

In this way, 16S rRNA gene-based profiling can be used to characterize the content of complex microbial populations, such as those found in the colon or vagina, and enables researchers to compare the relative compositions of microbial populations within an individual and among multiple individuals. Microbiota profiles from individuals who are healthy should resemble one another (i.e., be more closely related in terms of composition), whereas profiles from individuals suffering from a particular type of infection or disease should likewise resemble each other, but be different from the profiles of healthy individuals. Consequently, comparative analysis can serve to distinguish microbiota profiles among individuals and provide some diagnostic assessment of an individual's health status.

Cultivation-based studies took many years to complete a census for the human vaginal tract and came to the conclusion that Gram-positive *Lactobacillus* species were the dominant bacteria (present at over 90%) in most healthy women. It was believed that this *Lactobacillus*-dominant composition only changed during overt infection or symptomatic disease. The cultivation-independent studies using 16S rRNA gene-based microbial content analysis of the human vaginal tract showed no big surprises (Figure 5-2). *Lactobacillus* species were still the numerically dominant bacteria present in the population (at 80–90%), with the minor bacterial components being mostly other Gram-positive Firmicutes, such as *Staphylococcus* and *Streptococcus* species, *Gardnerella vaginalis,* and a few Gram-negative Proteobacteria, such

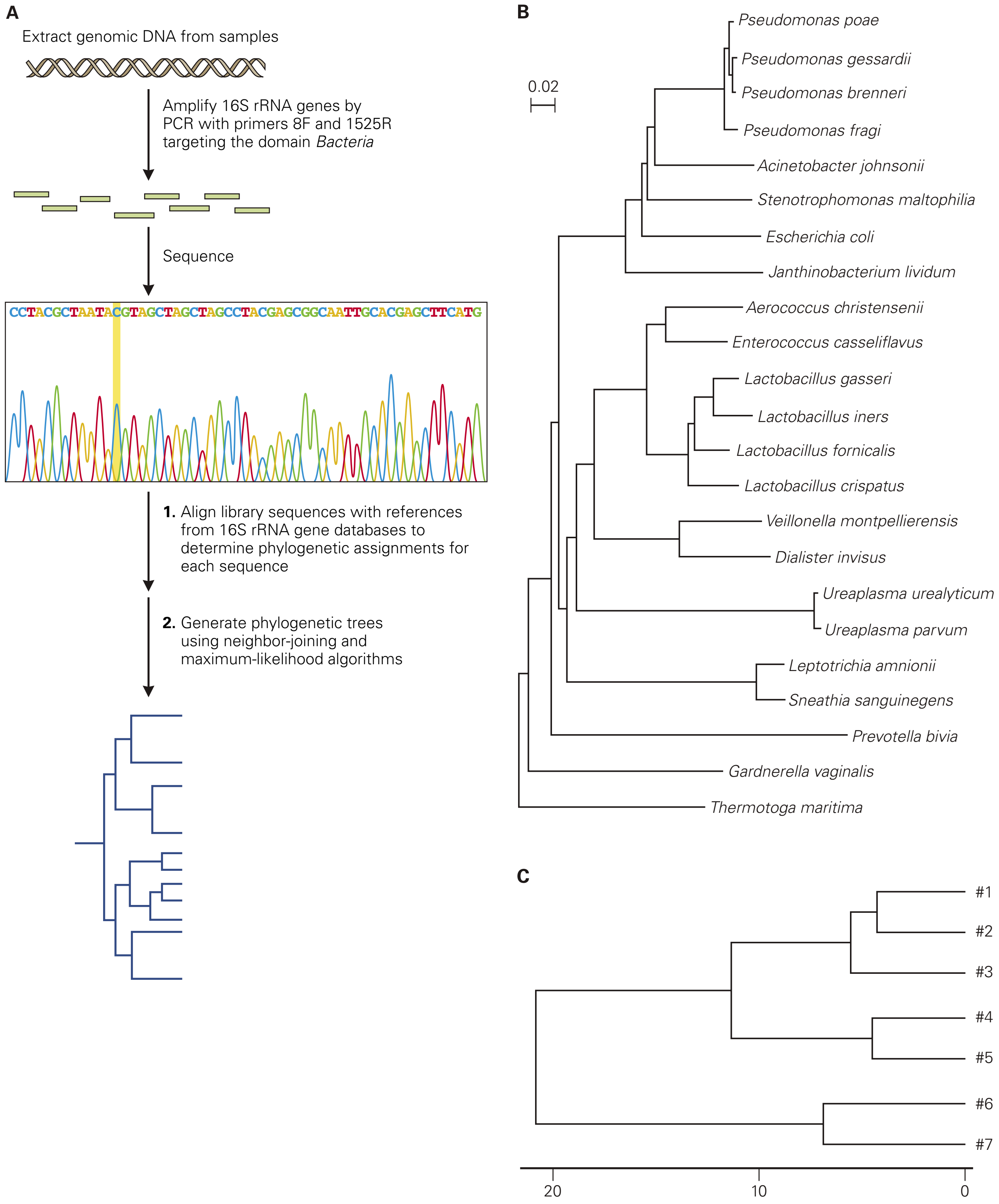
A
Extract genomic DNA from samples
Amplify 16S rRNA genes by PCR with primers 8F and 1525R targeting the domain *Bacteria*
Sequence
CCTACGCTAATACGTAGCTAGCTAGCCTACGAGCGGCAATTGCACGAGCTTCATG
1. Align library sequences with references from 16S rRNA gene databases to determine phylogenetic assignments for each sequence
2. Generate phylogenetic trees using neighbor-joining and maximum-likelihood algorithms
B
0.02
Pseudomonas poae
Pseudomonas gessardii
Pseudomonas brenneri
Pseudomonas fragi
Acinetobacter johnsonii
Stenotrophomonas maltophilia
Escherichia coli
Janthinobacterium lividum
Aerococcus christensenii
Enterococcus casseliflavus
Lactobacillus gasseri
Lactobacillus iners
Lactobacillus fornicalis
Lactobacillus crispatus
Veillonella montpellierensis
Dialister invisus
Ureaplasma urealyticum
Ureaplasma parvum
Leptotrichia amnionii
Sneathia sanguinegens
Prevotella bivia
Gardnerella vaginalis
Thermotoga maritima
C
#1
#2
#3
#4
#5
#6
#7
20
10
0

Figure 5-2. Phylogenetic trees to show relationships among microbial communities. Phylogenetic trees are used to illustrate ways rRNA gene sequence data can be displayed to show inferred evolutionary relationships among microbial communities based upon sequence similarities of the microbes with each other. (**A**) Shown are the steps used to generate a phylogenetic tree based on PCR amplification and sequencing of the nearly full-length 16S rRNA gene sequences (8–1,492) using the commonly used bacterial primer pair (27F + 1492R). **(B)** The phylogenetic tree shown here displays the phylogenetic relationships among the bacteria found in the vaginas of healthy women. The scale bar represents 0.02 nucleotide substitutions per site in the 16S rRNA gene sequences. **(C)** Shown is an example of a dendrogram (tree) of the phylogenetic relationships of the bacterial communities from seven different human vaginal microbiota samples, based on their 16S rRNA gene sequence profiles (similar to the one shown in panel B). The lines denote the phylogenetic distance between each of the samples, which is a measure of the relationship or similarity of one sample to the other. For example, samples #4 and #5 are about 10% different from each other (the lines, or branches of the tree, converge at around 5% on the bar index at the bottom), whereas samples #6 and #7 are about 40% different from all of the other samples (#1–#5).

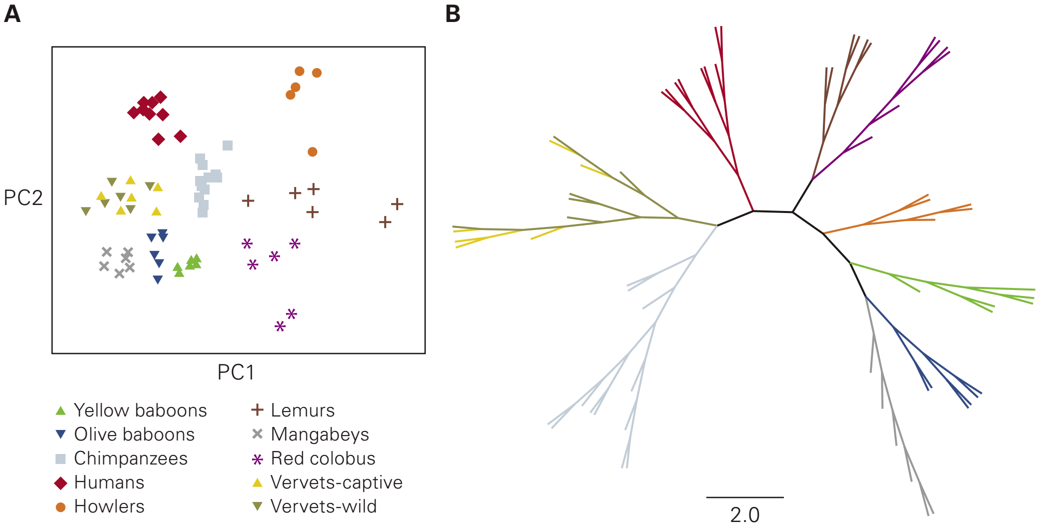

Figure 5-3. Principal component analysis to show relationships among microbial communities. A principal component analysis (PCA) plot is a tool for identifying patterns in data by highlighting their similarities and differences. The PCA plot is generated by a statistical program that uses orthogonal (perpendicular) transformation to convert the observed data (which fall within the area shown) into an ordinate plot. The first principal component (PC1) accounts for the largest variation in the data, which is attributed to factor 1, and the second principal component (PC2) accounts for the second-largest variation in the data, which is attributed to factor 2. Shown is a PCA plot **(A)** and the corresponding radial phylogenetic tree (a phylogenetic relationship tree that does not make assumptions about ancestry, in contrast to the trees shown in Figure 5-2) **(B)** based on 16S rRNA sequence profiles depicting the relationship among the vaginal microbiotas of humans and nonhuman primates. Vaginal samples are color-coded to match their host species. Data from Yildirim S, Yeoman CJ, Janga SC, Thomas SM, Ho M, Leigh SR; Primate Microbiome Consortium, White BA, Wilson BA, Stumpf RM. 2014. *ISME J* **8**:2431–2444.

as *E. coli* and *Pseudomonas* species. However, the phylogenetic DNA-based analysis did reveal a number of species-level differences in *Lactobacilli* content from the outcomes of cultivation-based studies, as well as considerable person-to-person, species-level variation in the non-*Lactobacilli* bacteria present. One finding that challenged some previous assumptions about what constitutes vaginal health was the finding that *G. vaginalis*, which previously was thought to be only associated with overgrowth during symptomatic **bacterial vaginosis (BV)**, is actually a common constituent of the normal microbiota of asymptomatic, apparently healthy women.

One question that a researcher might ask in developing an animal model of disease that manifests with a shift in the normal microbiota composition is if animals, such as laboratory rodents that are often used as models for human disease, have a microbiota that is similar to that of humans. And, if not, would another animal such as a nonhuman primate serve as a more appropriate animal model? To address this issue, a researcher might compare the 16S rRNA gene profiles of the microbiota at particular body sites in potential animal models. An example that may help you appreciate this decision process is provided by a study to determine whether baboons could serve as an appropriate animal model for studying the microbiota of the human vaginal tract.

Why baboons? The reason for choosing baboons is that anatomically, hormonally, and reproductively, the baboon and human uteruses and vaginas resemble each other closely, and both are clearly different from that of rodents, such that mice or rats might be considered to serve as poor models for human vaginal disease. Baboons have been widely used as an animal model in gynecological and reproductive studies of the female genital tract. Topics of these studies have ranged from endometriosis to the efficacy of birth control methods to menopause. Baboons are easier to house and more accessible to researchers than chimpanzees, another candidate for an animal model. Since the captive animals have the same diet and the same environment, this study also provided a chance to ask whether the considerable individual-to-individual differences seen in the human subjects were due to differences in environment, genetics, or other factors. However, in contrast to the microbiota of the human and rodent vaginal tracts, no culture-based analysis of the baboon microbiota had ever been done previously.

The 16S rRNA gene analysis of the baboon microbiota yielded a totally unexpected finding: the microbiota of the baboon vaginal tract was quite different from that of the human vaginal tract. Whereas *Lactobacillus* species dominate the human vaginal tract with minor components of other bacteria, species of Gram-positive bacteria (Firmicutes) other than *Lactobacillus* dominated the microbiota of the baboon vagina: mostly Clostridia, but also Gram-negative Fusobacteria and members of the phylum Bacteroidetes. This difference is illustrated in the clustering analysis shown in Figure 5-4. The difference between the composition of the human and baboon microbiota is striking, particularly in view of the fact that baboons seem to be so closely related to humans. Most of the bacterial species found in the baboon vaginal tract have representatives related to those isolated from humans, but largely those isolated from the human mouth and colon rather than the vaginal tract. Even within these groups, however, the human sequences clustered independently from the baboon sequences, indicating that, in many cases, the baboon sequences were not closely related to the human sequences and might represent new genera.

One question that immediately comes to mind considering these results is if the vaginal microbiotas of other nonhuman primates besides baboons are also different from that of humans. Indeed, a comparative 16S rRNA gene-based analysis of vaginal bacteria clearly demonstrated host species-specific vaginal microbial communities among humans and nonhuman primates (see Figure 5-3). Humans were distinct from other primates not only in microbial composition and diversity, but also because only humans possessed *Lactobacillus*-dominant vaginal microbiota. The reason for this interesting difference is still under investigation.

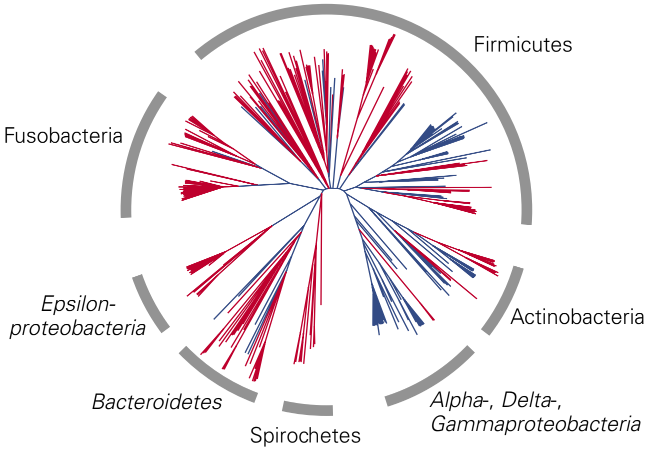

Figure 5-4. A radial phylogenetic tree showing the 16S rRNA gene sequence profiles of human and baboon vaginal microbes clustering to six major taxonomic groups. This radial tree shows relationships based on near-complete 16S rRNA gene sequences from the vaginal microbial community of captive baboons (red) and published human sequences (blue). Results show that the vaginal microbiota were clustered to six major taxonomic groups. Although Gram-positive bacteria dominate the vaginal microbiota of baboons, as they do in humans, there are major differences between the vaginal microbiotas of humans and baboons. Unlike humans, *Lactobacillus* species did not dominate the baboon microbiota. Even within the same taxa of bacteria, the baboon sequences clustered separately from the human sequences. Reprinted from Rivera AJ, Frank JA, Stumpf R, Salyers AA, Wilson BA, Olsen GJ, Leigh S. 2011. *Am J Primatol* **73:**119–129, with permission.

While phylogenetic relationship analysis based on 16S rRNA gene sequences has some clear advantages and can provide valuable insights regarding the microbiota composition, there are important limitations. In practice, given the complexity and diversity of the populations found in most parts of the body, a 16S rRNA gene analysis provides at best a representation of the most abundant genera and species in a particular site. This information, nevertheless, is extremely valuable because it narrows down the possible groups of bacteria in the population and can guide cultivation efforts. For example, the possible presence of anaerobic bacteria such as *Prevotella* species means that anaerobic conditions should be included in any attempt to cultivate members of the dominant groups.

A more serious limitation of this approach is that the data from a 16S rRNA gene analysis are only semiquantitative. Part of the problem is that endpoint PCR (see Figure 5-1B) is not strictly quantitative. This is due to the fact that some sequences seem to be amplified more efficiently than others (often referred to as PCR bias). Experience has shown that even some major members of the population may be misrepresented due to the absence of a strict quantitative representation of the microbiota. Nonetheless, the analysis gives an idea of what the leading members of the population are and provides a general assessment of their relative abundance.

Quantitative real-time PCR (qPCR). Once the members of a microbial community have been identified, the relative representation or abundance of those bacterial species can be determined by another method, called **quantitative real-time PCR (qPCR).** In one common variation of qPCR (Figure 5-5), genomic DNA from the microbial community is prepared and used directly as the template in PCR amplification reactions containing primer pairs that anneal specifically to the 16S rRNA genes of the bacterial species of interest in the population. The course of the PCR reaction is followed by an increase in fluorescence caused by intercalation of a dye, such as **SYBR green**, into the double-stranded PCR products. Alternatively, sequence-specific DNA probes labeled with the fluorescent dye at one end and a fluorescence quencher moiety at the other end can be used to detect the DNA of interest after hybridization of the labeled probe with its complementary sequence. The quencher is removed by the 5′ to 3′ exonuclease activity of the thermostable DNA polymerase, which allows for detection of fluorescence from the probe bound to its complementary DNA. Newer probe designs allow for enhanced specificity and sensitivity of detection, and when coupled with different fluorescent labels enable simultaneous detection of multiple target DNAs (**multiplexing**).

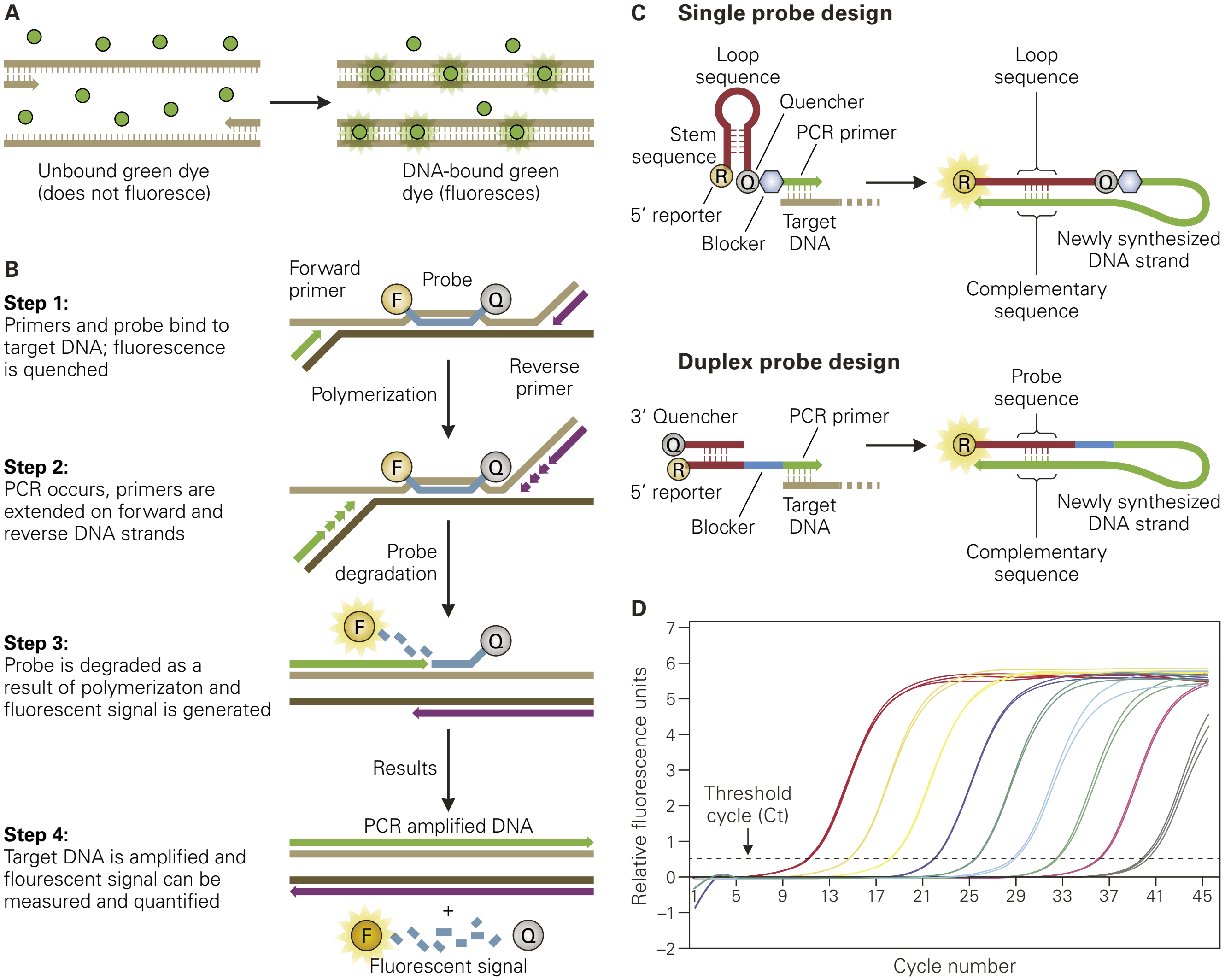
A
Unbound green dye
(does not fluoresce)
DNA-bound green
dye (fluoresces)
B
Step 1:
Primers and probe bind to target DNA; fluorescence is quenched
Forward primer
Probe
F
Q
Reverse primer
Polymerization
Step 2:
PCR occurs, primers are extended on forward and reverse DNA strands
F
Q
Probe degradation
Step 3:
Probe is degraded as a result of polymerizaton and fluorescent signal is generated
F
Q
Results
Step 4:
Target DNA is amplified and flourescent signal can be measured and quantified
PCR amplified DNA
+
F
Q
Fluorescent signal
C
Single probe design
Loop sequence
Quencher
Stem sequence
PCR primer
R
Q
5′ reporter
Blocker
Target DNA
R
Q
Loop sequence
Newly synthesized DNA strand
Complementary sequence
Duplex probe design
3′ Quencher
PCR primer
Q
R
5′ reporter
Blocker
Target DNA
R
Probe sequence
Newly synthesized DNA strand
Complementary sequence
D
Relative fluorescence units
7
6
5
4
3
2
1
0
−1
−2
Threshold cycle (Ct)
1
5
9
13
17
21
25
29
33
37
41
45
Cycle number

Figure 5-5. qPCR used to quantify specific bacteria in complex samples. Unlike conventional PCR, quantitative real-time PCR (qPCR) allows for detection of the target DNA in real time during the amplification process by measuring the fluorescence intensity above a given threshold. **(A)** Unbound fluorescent dye (typically SYBR green) does not fluoresce in the presence of ssDNA template, but does fluoresce when bound to newly formed dsDNA during the amplification process. **(B)** To eliminate fluorescence due to nonspecific binding of fluorescent dye, fluorescently labeled probes complementary to the target DNA template have quenchers attached that prevent fluorescence emission until the probes are degraded by the 5′ to 3′ exonuclease activity of the polymerase during the PCR amplification reaction. **(C)** Newer probe designs include fluorescence quenchers attached to the probe. Shown are two examples of such fluorescent dye-quencher probe designs: one as a single probe with step-loop structures to keep the fluorescent dye and quencher together and the other one as a duplex with one strand having the fluorescent dye and the other complementary strand having the quencher. The quenchers are removed during the second amplification cycle, such that the fluorescently labeled probe sequence can then hybridize to the complementary sequence on the newly synthesized target sequence. The blocker prevents the polymerase from extending the PCR primer. The fluorescence detected is directly proportional to the amount of target DNA present in the sample. **(D)** For qPCR analysis, the fluorescence is plotted versus the number of PCR thermocycles on a logarithmic scale, where the number of cycles at which the fluorescence is detected above the threshold is referred to as the threshold cycle (Ct), which is proportional to the amount of target DNA template in the sample.

For quantification, the qPCR procedure determines the kinetics of the increase in fluorescence intensity after each round of PCR amplification—this is the “real-time” aspect of the method—and relates these kinetics to a parameter called the threshold cycle number (Ct), which is inversely proportional to the starting concentration of the template DNA. Computer software analysis programs are then used to calculate the relative concentrations of each 16S rRNA gene, which is proportional to the relative number of bacteria of each species in the starting microbial community.

Relative quantification of target DNA can be determined based on comparison of the Ct obtained with that of an internal reference bacterium. Absolute quantification of a target bacterium in a sample can be determined by comparison of the Ct obtained with DNA standards using a calibration curve generated from titration of serial tenfold dilutions of a known concentration of the target bacterium.

Ultra-High-Throughput, Massively Parallel DNA Sequencing. A revolution has occurred within the last decade that has had a lasting and profound impact on profiling of microbial communities and on microbial functional genetics in general. Technological advances in ultra-high-throughput, massively parallel sequencing have taken place, which allow the simultaneous determination of multiple millions of base pairs of DNA sequences in single reaction runs (hence the term "massively parallel") at very reasonable costs. New bioinformatic methods now also allow the rapid and accurate assembly of these sequences into large regions of overlapping sequences (called **contigs**) that can be mapped into a complete genome.

At this writing, **Illumina sequencing technology** is the prevailing platform for massively parallel sequencing (Figure 5-6A). Despite the limitation of generating rather short read lengths of 50–300 contiguous bases, this method is extremely powerful and inexpensive in terms of capacity and time per sequencing run. At the time of this writing, the Illumina chemistry yields robust sequence determinations, even of long stretches of repeated bases, in as little as 6 hours for data outputs of 0.5–15 gigabases (1–25 million reads) to a few days for outputs of 7.5–35 GB (100–200 million reads). Meanwhile, improvements in DNA sequencing reagents and read detection technologies (Figure 5-6B) are appearing at an astounding pace. Modification to the original Illumina process for library preparation now enables **paired-end sequencing** (Figure 5-6C), which significantly improves alignment of reads to produce longer high-quality contigs that can be used for genome assembly.

An alternative sequencing platform that generates much longer sequence reads and is ideal for *de novo* genome sequencing is the **single-molecule, real-time (SMRT)** sequencing platform developed by **Pacific BioSciences (PacBio).** The PacBio SMRT system takes advantage of the intrinsic speed, fidelity (3′ to 5′ exonuclease activity), and importantly the high processivity of a single molecule of a special strand-displacing DNA polymerase, originally obtained from *Bacillus subtilis* phage phi29, to generate very long reads from a single molecule of DNA template (Figure 5-7). At this writing, each SMRT cell reaction run yields up to 5 GB of sequencing data in about 12 hours. Current chemistry and library preparation advances have pushed the limits of sequence read lengths up to 60 kB (with average read lengths of 10–20 kB), which can yield large overlapping contigs of up to 15 MB in length, enabling the complete assembly of most microbial genomes, including plasmids, without gaps.

Currently, the Illumina and PacBio sequencing platforms are often used in combination to determine the complete genome sequence of a new bacterial isolate. If a complete genome is already available for a closely related bacterium such that that genome can be used as a template, then sequence assembly of overlapping contigs from a new bacterial isolate can readily be completed without any or only a few gaps remaining to be filled. For this, the shorter reads obtained by the Illumina platform are sufficient. For *de novo* genome sequencing (where no existing complete genome is available for a related bacterium), the longer reads of the PacBio platform provide a scaffold and high-quality draft sequence that can be rapidly polished by the high accuracy and **coverage** (i.e., the large number of overlapping sequence reads used to accurately call a sequence) provided by the Illumina sequencing. As these DNA sequencing methods develop, it will become increasingly quick and cost-effective to not only take the census of bacterial species in complex microbial communities, but also to sequence parts or entire genomes directly from the mixture of genomic DNA isolated from these populations.

To understand the power of current **whole-genome sequencing technologies**, let us consider a common, recurring problem in bacterial genetics. Interesting mutations often arise spontaneously in bacteria whose complete genomes have already been determined. Researchers are often interested in knowing what these mutations are, particularly if the bacterium is a pathogen. Classical bacterial genetics provides exceedingly clever ways to map mutations so that they can be located by conventional sequencing of a limited region of the chromosome of the mutant strain. However, these classical methods are often time-consuming and are far from foolproof, especially in bacterial species lacking powerful genetic systems, such as many bacterial pathogens.

For example, the Illumina sequencing technology is routinely used to locate mutations in bacteria, such as *Streptococcus pneumoniae*, whose genome contains about 2.2 million base pairs. In this case, chromosomal DNA isolated from mutant strains is sheared into random fragments of about 500 base pairs. Adaptors required for hybridization during the sequencing method are ligated to the ends of the DNA fragments, and the resulting products are amplified by PCR to give random libraries of genomic fragments from each mutant. The adaptors used for each mutant genome have slightly different sequences ("**barcodes**"), so that DNA sequences from more than one mutant can be sequenced simultaneously and later distinguished. The resulting barcoded libraries are mixed and subjected to Illumina sequencing. Currently, with the highest-capacity instruments, such a sequencing run yields about 120 billion bases of sequence comprised of approximately 400 million reads with

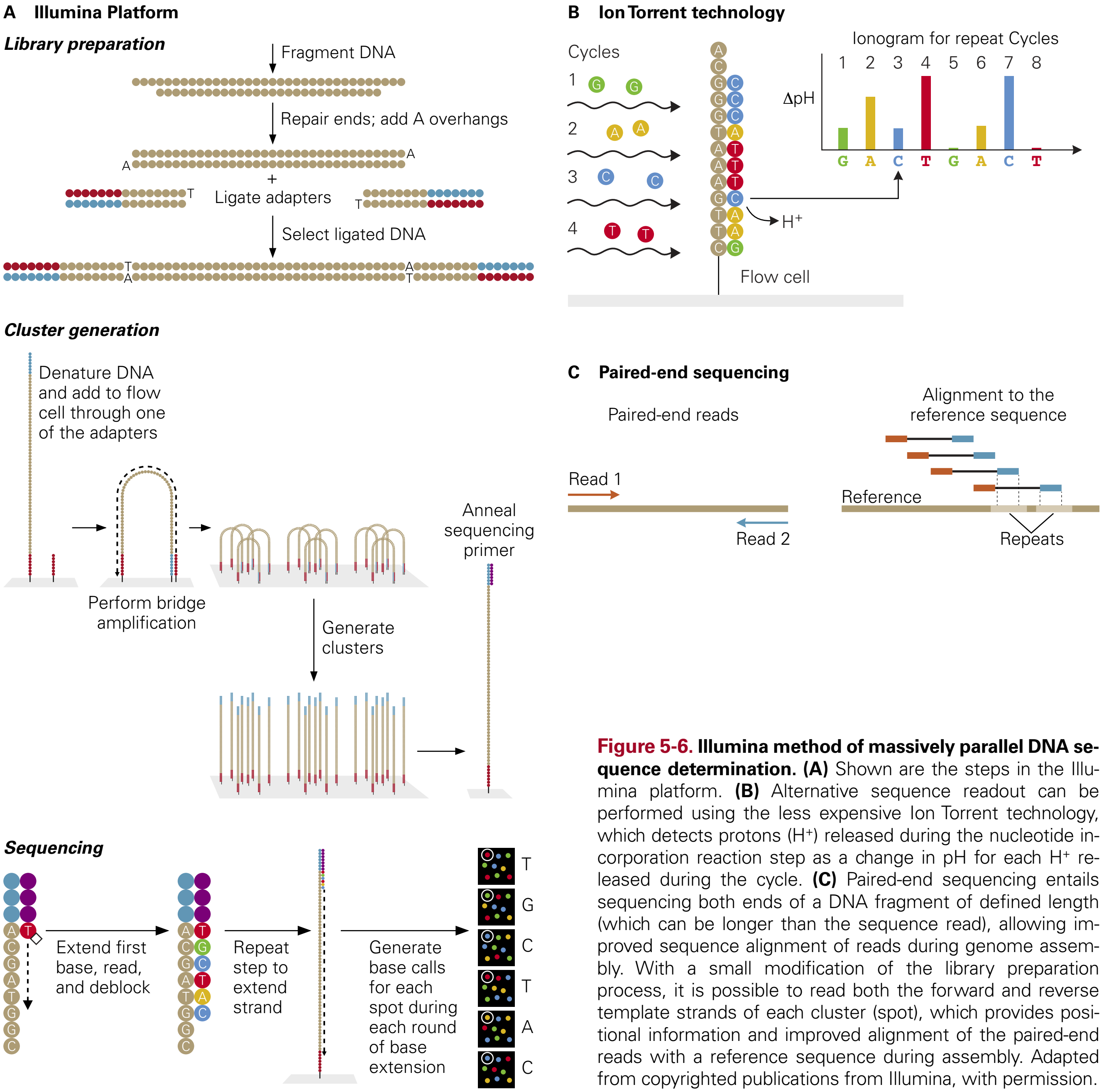
A Illumina Platform
Library preparation
Fragment DNA
Repair ends; add A overhangs
A
A
+
T
Ligate adapters
T
Select ligated DNA
T
A
A
T
Cluster generation
Denature DNA and add to flow cell through one of the adapters
Perform bridge amplification
Anneal sequencing primer
Generate clusters
Sequencing
Extend first base, read, and deblock
Repeat step to extend strand
Generate base calls for each spot during each round of base extension
T
G
C
T
A
C
B Ion Torrent technology
Cycles
1
2
3
4
G
A
C
T
Ionogram for repeat Cycles
1 2 3 4 5 6 7 8
ΔpH
G A C T G A C T
H+
Flow cell
C Paired-end sequencing
Paired-end reads
Read 1
Read 2
Alignment to the reference sequence
Reference
Repeats

Figure 5-6. Illumina method of massively parallel DNA sequence determination. (A) Shown are the steps in the Illumina platform. **(B)** Alternative sequence readout can be performed using the less expensive Ion Torrent technology, which detects protons (H^+) released during the nucleotide incorporation reaction step as a change in pH for each H^+ released during the cycle. **(C)** Paired-end sequencing entails sequencing both ends of a DNA fragment of defined length (which can be longer than the sequence read), allowing improved sequence alignment of reads during genome assembly. With a small modification of the library preparation process, it is possible to read both the forward and reverse template strands of each cluster (spot), which provides positional information and improved alignment of the paired-end reads with a reference sequence during assembly. Adapted from copyrighted publications from Illumina, with permission.

lengths of up to 300 bases! Thus, the base-pair coverage of a single *S. pneumoniae* genome in a reaction lane would be about 27,000-fold in this example. This extraordinarily high level of coverage means that barcoded, fragmented genomic libraries from 27 different mutants could be sequenced simultaneously at 1,000-fold coverage in a single sequencing run in less than a week. Subsequent bioinformatic sequence comparisons with the known genome sequence of *S. pneumoniae* could then reveal the locations of single-nucleotide point mutations, small and large deletions, or even regions of chromosomal duplication that contribute to the mutant phenotypes. And what is the cost? At this writing, the cost of the sequence determination and bioinformatic analyses per mutant genome is far less than the cost of classical genetic approaches, which previously required weeks to months to obtain and, if they worked, would yield far less information compared to less than one week for the large-scale sequencing analysis.

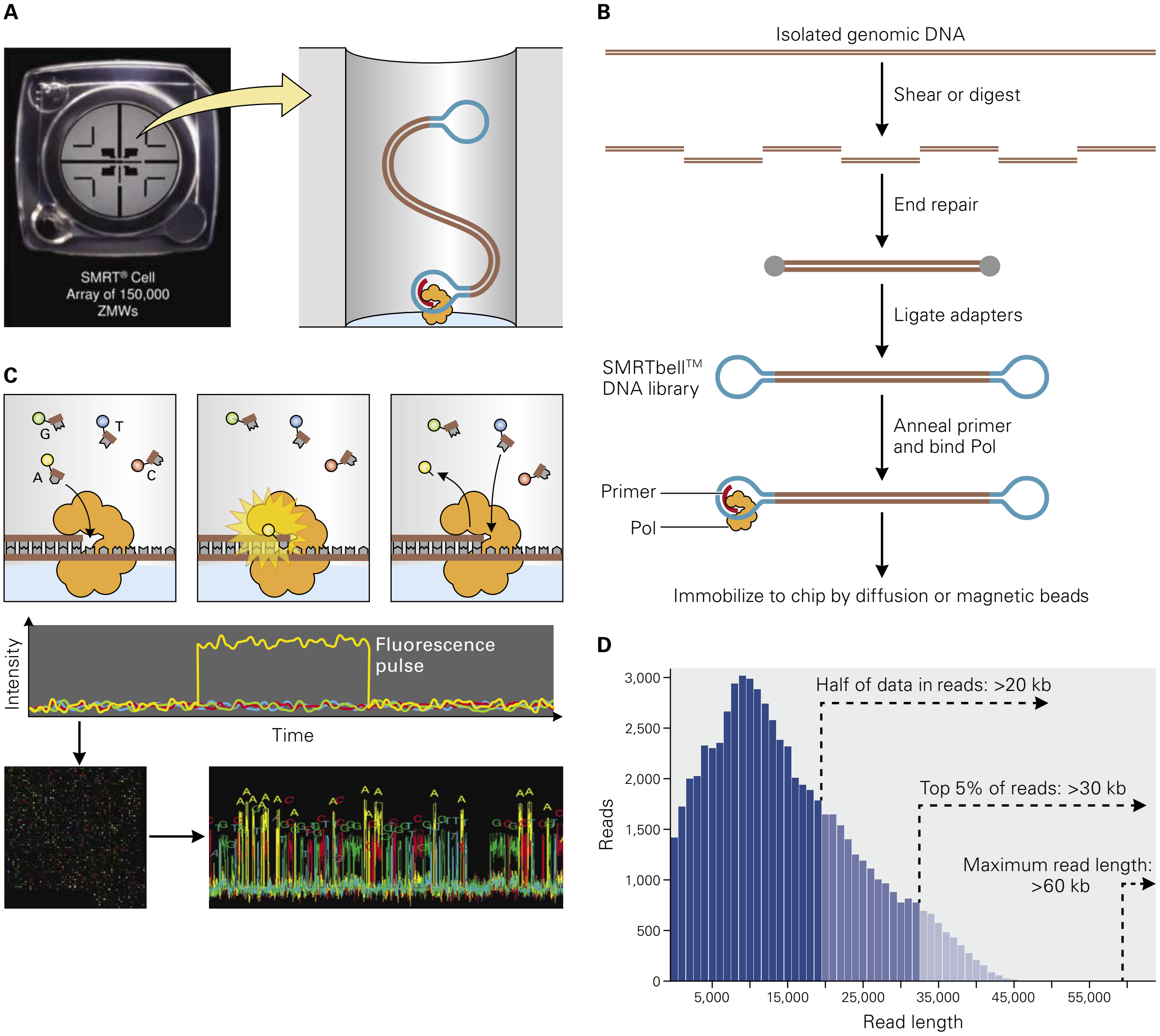
A
SMRT® Cell
Array of 150,000
ZMWs
B
Isolated genomic DNA
Shear or digest
End repair
Ligate adapters
SMRTbell™
DNA library
Anneal primer
and bind Pol
Primer
Pol
Immobilize to chip by diffusion or magnetic beads
C
G
T
A
C
Intensity
Fluorescence
pulse
Time
D
Half of data in reads: >20 kb
Top 5% of reads: >30 kb
Maximum read length:
>60 kb
Reads
3,000
2,500
2,000
1,500
1,000
500
0
5,000
15,000
25,000
35,000
45,000
55,000
Read length

Figure 5-7. The PacBio SMRT sequencing platform. The Pacific Biosciences (PacBio) single-molecule, real-time (SMRT) sequencing technology uses a single highly processive, strand-displacing DNA polymerase that is immobilized to the base of a matrix in a fluorescence-detection well. **(A)** Each SMRT reaction cell contains 150,000 zero-mode waveguides (ZMWs), which are nanophotonic confinement wells that are illuminated from below and have a detection volume of 20 zeptoliters (10^{-21} liters). The technology uses ZMWs to observe the base incorporations of an anchored polymerase molecule, which allows light to illuminate only the bottom of the well, in which a single DNA polymerase plus DNA template complex is immobilized. **(B)** The current version of the SMRT technology uses SMRTbell template preparation to generate a circularized, double-stranded DNA template from appropriately sized fragments of the genomic DNA, generated either by random shearing or by amplification of target regions of interest. Universal hairpin adaptors are then ligated onto the ends of the DNA fragment to generate the SMRTbell library. These hairpin dimer sequences are removed at the end of the library preparation protocol. The sequencing primer is then annealed to the SMRTbell template, followed by binding of the DNA polymerase, to form a complex, which is then immobilized to the bottom of the ZMW well. **(C)** In each ZMW well, a single molecule of the DNA polymerase sequences a single nucleotide at a time of a single molecule of DNA template. Phospholinked nucleotides, each labeled with a different fluorescently linked dye, are introduced into the ZMW well. As a nucleotide binds the polymerase-DNA complex in the ZMW well, the fluorescently labeled phospholinked nucleotide emits a light pulse that is detected. When the phosphodiester bond is cleaved during the DNA elongation reaction, the fluorescent dye is released and diffuses away from the ZMW detection zone and the light emitted by that nucleotide is no longer detected. The fluorescent read output from incorporation of the four different fluorescent dyes is captured for the entire SMRT cell over time and processed by a computer into sequence reads (one read per ZMW well). **(D)** With the latest chemical reagent kits, SMRT technology can provide real-time sequence reads of 5–60 kB in length. Adapted from copyrighted publications from PacBio, with permission.

16S rRNA Gene-Based Profiling of Complex Microbial Communities. Scientists are often interested in understanding the effect of certain conditions or factors over time on composition of the microbial community. For instance, a researcher might be interested in the effect of diet, hormones, or age on the composition of the microbiota, or how antibiotic therapy impacts the microbiota. To accomplish this, it is necessary to collect at any given time a sample of the microbes that is representative of the whole community (a profile) such that changes in abundance or diversity can be monitored.

One profiling method involves DNA microarray chips, called **phylochips**, which are comprised of tens of thousands of oligonucleotide-containing spots, each corresponding to a set of taxa-defining sequences, including rRNA gene regions and other unique gene sequences that can distinguish among the various microbial species (taxa or "**phylotypes**") present in the sample of interest (Figure 5-8). To design appropriate phylochips, the microbial species present in environmental niches, including different areas of the human body, must first be identified by rRNA gene sequencing methods from a representative sample. Oligonucleotide probes that will hybridize to the rRNA genes from each species are then printed onto the microarray. These phylochips can be used to monitor shifts in microbial community compositions in environmental and clinical samples. The greatest advantage of this phylochip technology is that all microorganisms of interest in an entire community, not just bacteria, can be detected in a single assay by multiple probes to give reliable taxonomic information.

The current limitation of these phylochips is that they first require the availability of the rRNA gene and other key distinguishing gene sequences for identification of each of the microbes expected to be present in the samples. But the increasing number of powerful high-throughput sequencing technologies currently available at ever more affordable costs, as well as the vast number of microbial sequences already deposited into the DNA databases, makes the design of phylochips for various microbial and clinical ecosystems increasingly feasible. Indeed, the breathtaking advances in massively parallel DNA sequencing methods described previously now allow cost-effective studies of the changing dynamics of complex microbial communities over time and under different conditions by direct sequencing alone.

Comparative 16S rRNA Gene-Based Profiling of Complex Microbial Communities. Current massively parallel sequencing technologies allow researchers to sequence millions of 16S rRNA genes per sample and multiple samples simultaneously, enabling microbial profiles for multiple samples to be rapidly generated and compared. Because of the depth of sequencing obtained from these large numbers of reads, these profiling methods enable researchers to rapidly assess differences in patterns between complex microbial communities, thereby gaining insight into the diversity or dynamics of the microbial communities.

It should be noted, however, that only nearly complete 16S rRNA sequences provide accurate measures of taxonomic identity and diversity within a microbial community. Unfortunately, while current sequencing technologies have made microbial community profiling routine and affordable, this has come at the expense of read length: sequence reads typically cover only a portion (usually 250–600 nucleotides) of the ~1.5-kB 16S rRNA gene, necessitating that researchers choose shorter regions, such as V1–V3, V3–V4, V4–V5, or V3–V5 (see Figure 5-1A), for sequence analysis. Consequently, this compromises the resolution of the taxonomic identification and phylogenetic relationship determinations and should be taken into account when interpreting microbial sequencing data.

Despite the previously mentioned limitations, profiling of microbiotas based on shorter variable regions of the 16S rRNA gene using current sequencing technologies has proven invaluable in providing insights into the role of microbiota in health and disease and how our microbiotas change over time and under different conditions. For instance, such profiling analysis using species-level classification has been used to compare the vaginal microbiota of women of African-American versus European ancestry (Figure 5-9). The results revealed significant differences in the microbiota composition of these two groups of women. The profiles confirmed that women of European ancestry are more likely to have a *Lactobacillus*-dominated vaginal microbiota, whereas African-American women are more likely to have a more diverse vaginal microbiota, similar to a composition often found in women displaying symptoms of BV. Even at the species level, significant differences were observed within the *Lactobacillus* genus, with women of European ancestry more likely to harbor *L. crispatus, L. jensenii,* and *L. gasseri*. These findings supported other studies indicating that African-American women are more prone to having diseases that are associated with BV, such as increased risk of sexually transmitted diseases and adverse pregnancy outcomes. Researchers are now using this information to understand the factors that dictate these differences and how to develop treatment options that take these differences into account.

Tracking Microbes Through Space and Time by Multilocus Sequence Typing (MLST) and Whole-Genome-Sequencing. We have considered the problem of identifying different bacterial species in complex mixtures taken from the environment or from sites in the human body. But scientists and epidemiologists are often faced with the problem of distinguishing different isolates or variants of a single

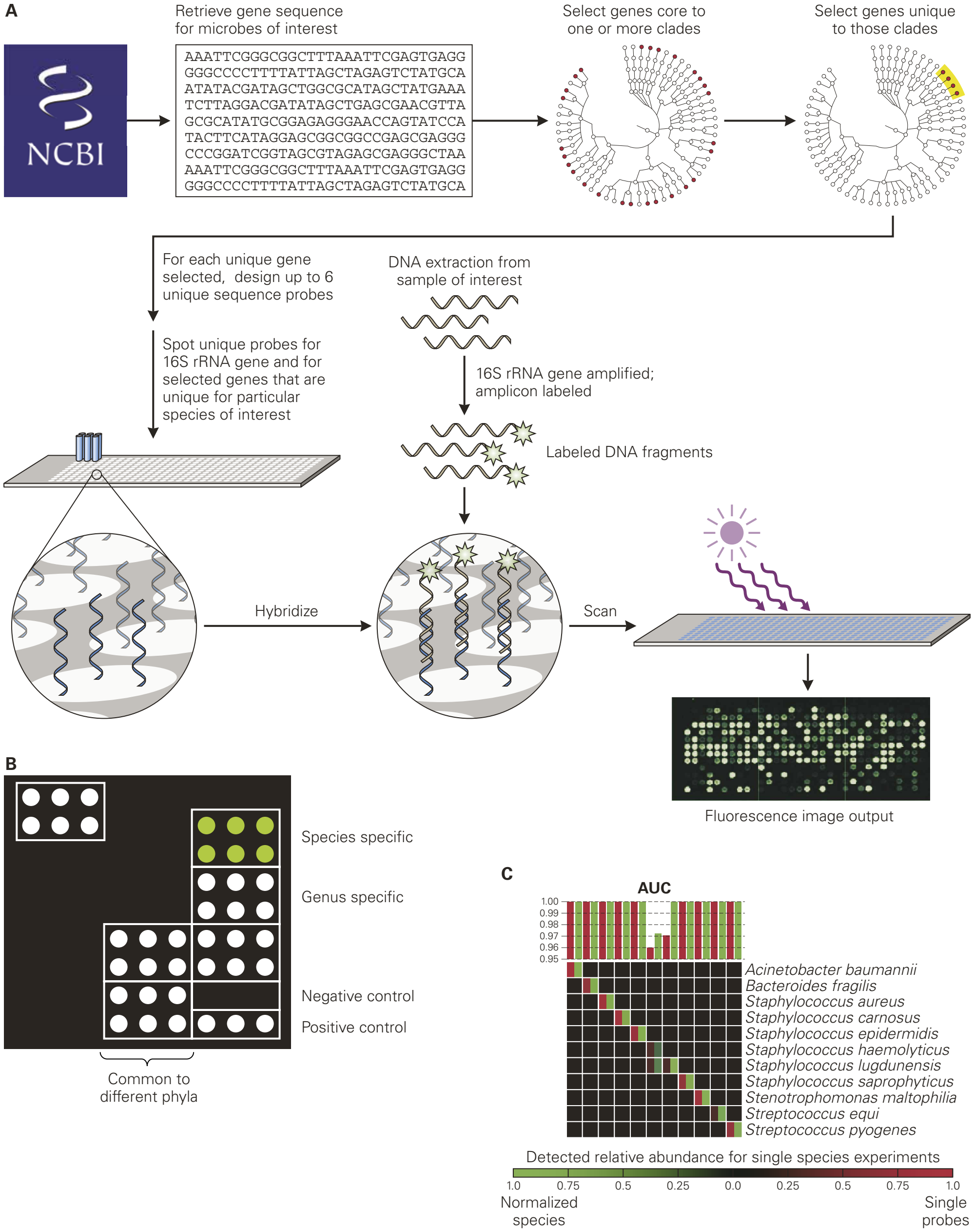
A
NCBI
Retrieve gene sequence for microbes of interest
AAATTCGGGCGGCTTTAAATTCGAGTGAGG
GGGCCCCTTTTATTAGCTAGAGTCTATGCA
ATATACGATAGCTGGCGCATAGCTATGAAA
TCTTAGGACGATATAGCTGAGCGAACGTTA
GCGCATATGCGGAGAGGGAACCAGTATCCA
TACTTCATAGGAGCGGCGGCCGAGCGAGGG
CCCGGATCGGTAGCGTAGAGCGAGGGCTAA
AAATTCGGGCGGCTTTAAATTCGAGTGAGG
GGGCCCCTTTTATTAGCTAGAGTCTATGCA
Select genes core to one or more clades
Select genes unique to those clades
For each unique gene selected, design up to 6 unique sequence probes
Spot unique probes for 16S rRNA gene and for selected genes that are unique for particular species of interest
DNA extraction from sample of interest
16S rRNA gene amplified; amplicon labeled
Labeled DNA fragments
Hybridize
Scan
Fluorescence image output
B
Species specific
Genus specific
Negative control
Positive control
Common to different phyla
C
AUC
1.00
0.99
0.98
0.97
0.96
0.95
Acinetobacter baumannii
Bacteroides fragilis
Staphylococcus aureus
Staphylococcus carnosus
Staphylococcus epidermidis
Staphylococcus haemolyticus
Staphylococcus lugdunensis
Staphylococcus saprophyticus
Stenotrophomonas maltophilia
Streptococcus equi
Streptococcus pyogenes
Detected relative abundance for single species experiments
1.0
0.75
0.5
0.25
0.0
0.25
0.5
0.75
1.0
Normalized species
Single probes

Figure 5-8. **Phylochips for microbial-community profiling.** Current phylochip platforms are a microarray-based method that taxonomically identifies microbes in a given sample through hybridization of the rRNA gene in that sample to all nine of the variable regions of the 16S rRNA gene. **(A)** General procedure for making a phylochip. Complete genomes are retrieved from the National Center for Biotechnology Information microbial database and a phylogenetic relationship tree is generated. Genome comparison of bacteria within each clade (group of related bacteria) is used to identify core gene sequences that are shared by most members of the clade. After sequence alignment with all other bacterial and archaeal genomes, core gene sequences that are unique to each target clade (species) are identified, and DNA probes are designed against up to 10 of the unique genes for each target species. **(B)** Example of how a phylochip could be used to distinguish microbes at the genus and species levels in a sample. Positive and negative controls are included to ensure that the hybridization steps worked and that there is no background detection, respectively. Some spots are probes targeted toward distinguishing specific microbes at the genus level (nine spots with probes for each of the V1–V9 variable regions of the 16S rRNA genes), while other spots are probes targeted toward distinguishing specific microbes at the species or subspecies level (spots corresponding to unique genes found only in particular clades or subspecies). Custom probes (upper left corner of figure) can be made for identifying species with particular genes present (e.g., virulence factors). **(C)** Heat map showing the intensities for individual probes (red) and total species probes (green) in the bacterial sample. A plot of the area under the curve (AUC) shows the relative abundances of each of the bacterial species in the sample.

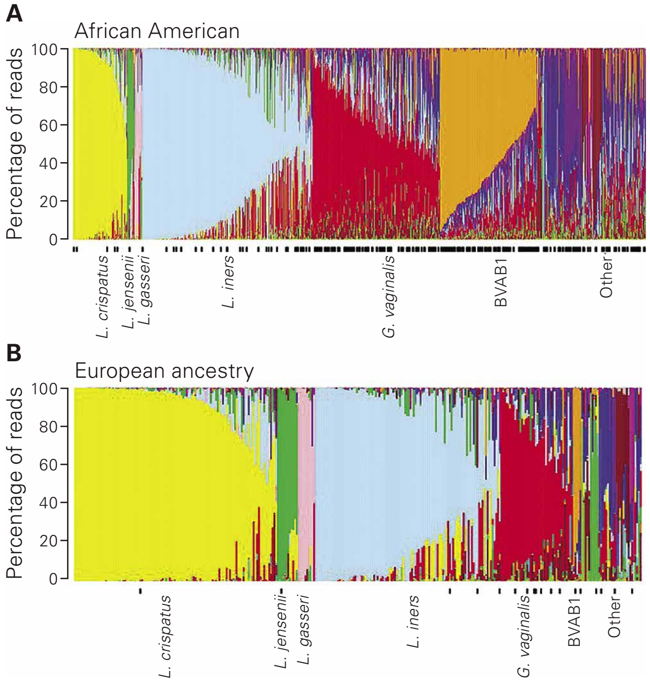

Figure 5-9. Comparative 16S rRNA gene-based profiling of bacterial communities from women of African-American versus European ancestry. Relative abundance profiles of vaginal bacteria using species-level classification from **(A)** 960 African-American women and **(B)** 330 women of European ancestry enrolled in the Vaginal Human Microbiome Project. Each vertical line represents the bacterial composition profile from one vaginal sample, where the profiles are grouped by the dominant species into different profile types. The colors correlate to different bacterial species: yellow = *Lactobacillus crispatus*; green = *Lactobacillus jensenii*; pink = *Lactobacillus gasseri*; light blue = *Lactobacillus iners*; red = *Gardnerella vaginalis*; orange = uncultivated clostridial species associated with bacterial vaginosis type 1 (BVAB1); dark blue = *Prevotella* species. The black bars underneath the plot indicate microbial profiles from women diagnosed with bacterial vaginosis (BV). Reprinted from Fettweis JM, Brooks JP, Serrano MG, Sheth NU, Girerd PH, Edwards DJ, Strauss JF 3rd; Vaginal Microbiome Consortium, Jefferson KK, Buck GA. 2014. *Microbiology* **160(pt10)**:2272–2282, with permission.

bacterial species. Suppose that there is an outbreak of *Staphylococcus aureus* in a hospital, and you need to trace how this particular strain got into and around the hospital. Was it carried by a hospital worker or by a family member of a patient? Or suppose there is an outbreak of food poisoning caused by *Listeria monocytogenes*, which can contaminate food-processing equipment and ready-to-eat meat products. You want to find out how this strain of *L. monocytogenes* entered the food chain and whether it is similar to strains that caused previous outbreaks. The rRNA genes of all isolates of *S. aureus* have the same sequence, because the rRNA genes change very slowly within a given species. Likewise, the DNA sequences of the rRNA genes are nearly identical for different variants and isolates of *L. monocytogenes*.

Clearly, 16S rRNA gene-based strain typing will not work to trace infections such as these. But unlike rRNA genes, the DNA sequences of genes that encode housekeeping enzymes and virulence factors do change with time within a species. That is, isolates from *S. aureus* or *L. monocytogenes* from different locations or sources accumulate slight differences in the sequences in their housekeeping and virulence factor genes over time. This drift arises partly because the genetic code is degenerate. Recall that more than one codon can specify the same amino acid (e.g., there are six specifying leucine), and much of this redundancy occurs at the third positions of codons. Therefore, the DNA sequences of housekeeping genes can show variations in different isolates of a given bacterial species and still specify the same amino acid in the enzyme product. The drift in virulence factor genes often involves changes in amino acids compared to those in housekeeping genes, because the virulence factor genes are subjected to strong selective pressures during infection.

This variation within coding sequences is the basis of **multilocus sequence typing (MLST)**, but rather than relying on genetic drift in just one housekeeping or virulence gene, the DNA sequences of regions in multiple (usually seven or more) genes are analyzed for each isolate. For each of the genes selected for a particular bacterial species, the different sequences are assigned as alleles. MLST analysis using multiple genes is easy and has become cost-effective recently. Bioinformatics is used to identify regions of seven or more housekeeping or virulence genes that show variations in a given bacterial species. Pairs of PCR primers are designed to amplify and sequence about 500 base pairs from each of these variable regions. The sequences of these multiple loci can then be compared among samples or with other isolates in databases to trace the relatedness of the different isolates locally and worldwide. This analysis can also take into account combinations of alleles in bacterial species that exchange genetic material and undergo horizontal gene transfer frequently.

Going back to our examples, samples collected from staff, visitors, patients, and locations in the hospital could be cultured to identify those that contain *S. aureus*, which is a common commensal bacterium that is easily identified on growth medium. The different isolates of *S. aureus* would be subjected to MLST analysis. Progeny or clonal isolates will have the same DNA sequences in most of the multiple loci, whereas strains from a different source may have loci with sequence variations. The resulting profile would indicate whether patients are infected with the same strain of *S. aureus* and where this strain may have arisen in the hospital. Similarly, MLST analysis can trace the sources of *L. monocytogenes* through the chain of food preparation of the outbreak mentioned earlier as well as previous outbreaks and thereby identify the root of the contamination problem.

MLST analysis does not distinguish whether multiple sequence differences observed between alleles is a result of multiple point mutations or a single recombination event. Consequently, MLST analysis does not assign a higher similarity value to sequences differing by a single nucleotide compared to sequences with multiple nucleotide differences. To determine phylogenetic relationships of closely related species with high clonal evolution, **multilocus sequence analysis (MLSA)** is used, in which the selected housekeeping and virulence gene sequences are first **concatenated** (i.e., virtually linked in tandem to each other) before performing comparative phylogenetic analysis. This process provides greater discriminatory power for determining phylogenetic relationships.

MLSA analysis has recently been greatly extended by using whole-genome sequence determinations, rather than sequences of a limited number of housekeeping or virulence genes. MLSA by whole-genome sequencing provides higher resolution for differentiating bacterial isolates and for tracking the evolution and spread of virulent and/or antibiotic-resistant bacteria. As an example of the impact that whole-genome sequencing has had on MLSA, let us consider carbapenem-resistant *Klebsiella pneumoniae*, which has emerged as a serious clinical problem in hospitals. Previously, the vast majority of resistant clinical isolates had been genetically characterized by MLST as a single multilocus type (designated as ST258), leading to the hypothesis that a single clone of ST258 was responsible for the global spread of these multidrug-resistant bacteria. However, whole-genome sequencing and phylogenetic analysis of a large number of ST258 clinical isolates from diverse geographic locations revealed unexpected diversity among the isolates, including divergence into two distinct genetic clades due to a 215-kB region that appears to be a hotspot for DNA recombination events. This region also contains genes involved in capsule polysaccharide biosynthesis that likely contributed to the observed serological variation. In another example, phylogenetic relationship analysis based on whole-genome sequencing of 132 clinical isolates from around the world was used to track the recent evolutionary history of the **dysentery** (bloody diarrhea) pathogen *Shigella sonnei* from Europe to other parts of the world (Figure 5-10).

Characterizing Microbiomes by Using Metagenomic Analysis

The various rRNA gene sequencing approaches mentioned previously only give information regarding what types of microbes are present in a community. A limitation of this type of approach is its failure to generate functional genomic information for deciphering the metabolic contributions of the microbes present in an ecosystem. For example, it does not provide direct information about which microbes might have a symbiotic relationship by producing and secreting metabolic products that could cross-feed other microbial species or the host. The current state of mega-scale DNA sequencing technology has spurred interest in a more ambitious approach to analyzing the body's microbiota: determining the sequences of all of the individual microbial genomes of the body (the microbiomes). The goal of this approach, called **metagenomic analysis**, is designed to go beyond the question of cataloging what organisms are present (i.e., the census question) to the question of what the metabolic and physiologic potential of the microbiota is (i.e., the metabolic genes and pathways that are present).

But what can be done about species whose genomes are incomplete or not in the database at all? To answer this question, the next stage of metagenomic analysis involves isolation and mega-scale sequencing of all genomic DNA from an entire mixed microbial population (called the **metagenome**) so as to harvest the remarkable and vast diversity present. In fact, the advances in robotics, ultra-high-throughput, massively parallel sequencing, and bioinformatics assembly technologies are already leading to determination of complete genomes of microbes directly (without cultivation or cloning of individual isolates) from the mixed genomic DNA samples isolated from complex microbiota communities. For instance, metagenomic analysis can be applied to profiling strain-level variation in microbial communities (Figure 5-11).

Assembling individual genome sequences from many thousands of sequences is still challenging for existing bioinformatics programs, but here again, rapid advances are being made in the analysis of the huge volume of new sequence data that is emerging, and these advances are beginning to make what seems unimaginable today feasible tomorrow (in biotechnology, as in most modern scientific endeavors, "impossible" just means it is "not possible *yet*"). Interpretation of a metagenomic analysis is much more complex than 16S rRNA analysis, or even a whole genome assembly and analysis, and details are beyond the scope of this textbook, but for the adventurous, several examples of recent metagenomic analyses of human microbiotas are provided in the suggested readings. What these analyses allow us to do is have a glance at the types of biosynthetic and metabolic pathways that the microbes in any given population might have at their disposal to utilize.

To help tackle the daunting task of defining our microbiomes, the National Institutes of Health launched the **Human Microbiome Project (HMP)** in 2007 with the objective of sequencing the collective genomes of all of the microbes (bacteria, archaea, fungi, protozoa, and viruses) that comprise the microbiotas of five targeted sites in the human body: the skin, mouth, nasal passages/lungs, vagina, and gut. The goal of the HMP was to lay the groundwork

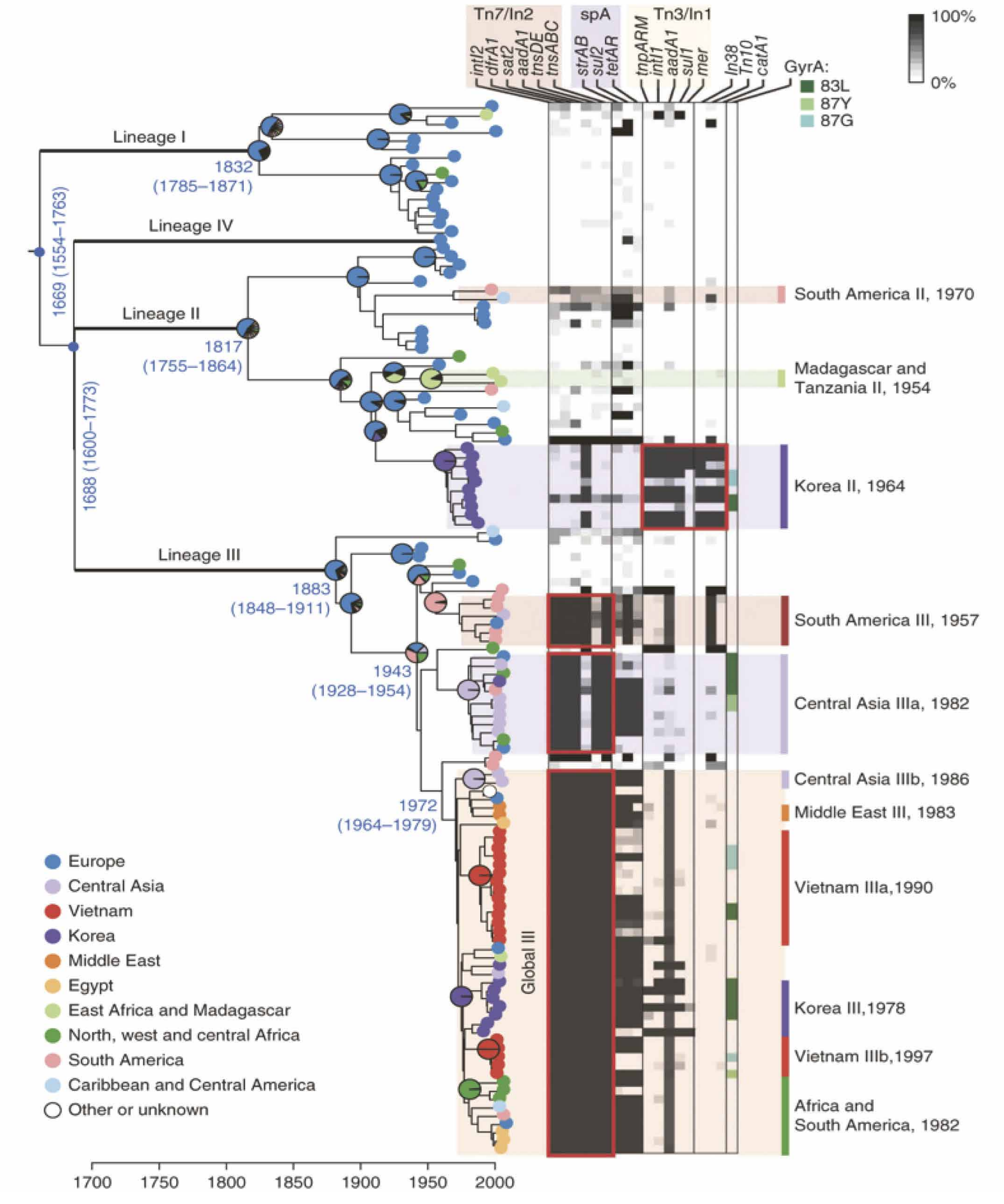

Figure 5-10. Phylogenetic relationship analysis using comparative whole-genome sequence profiling to track the evolution and dissemination of the dysentery pathogen *Shigella sonnei*. The dysentery pathogen *Shigella sonnei* was once predominant in developed countries, but it is now emerging as a major problem in developing countries. Whole-genome sequencing of 132 globally distributed clinical isolates, followed by phylogenetic analysis, showed that the current *S. sonnei* strains descended from a common ancestor in Europe less than 500 years ago. The results also showed that by the late 19th century, *S. sonnei* had diverged into four distinct lineages with strong regional clustering. The heat map shows the distribution of genes associated with antibiotic resistance. Known antibiotic-resistance mutations in the gene encoding DNA gyrase, GyrA, are indicated by color. Probable multidrug-resistance (MDR) gene acquisition events are boxed in red. Geographically localized clonal expansions are highlighted with their median estimated divergences dates. Reprinted from Holt KE, Baker S, Weill FX, Holmes EC, Kitchen A, Yu J, Sangal V, Brown DJ, Coia JE, Kim DW, Choi SY, Kim SH, da Silveira WD, Pickard DJ, Farrar JJ, Parkhill J, Dougan G, Thomson NR. 2012. *Nat Genet* **44:**1056–1059, with permission.

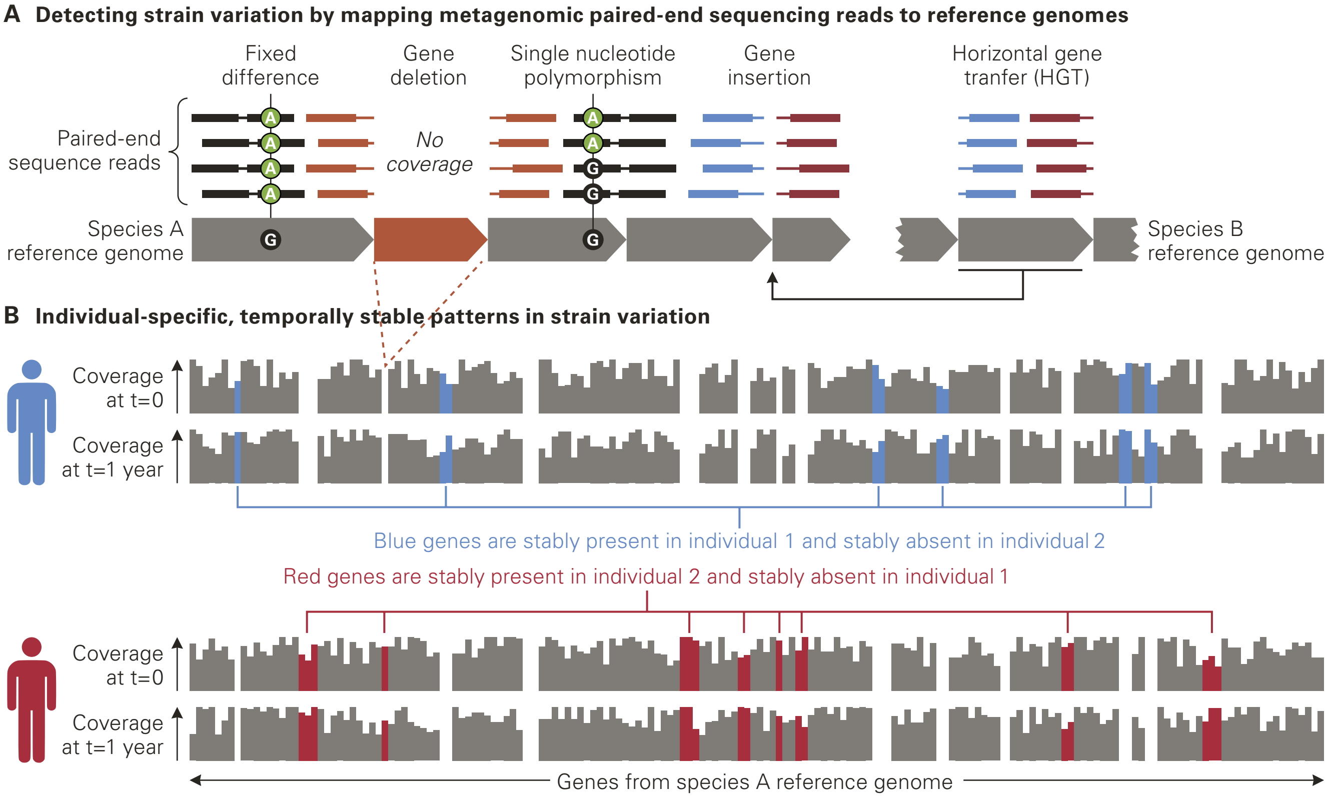
A Detecting strain variation by mapping metagenomic paired-end sequencing reads to reference genomes
Fixed difference
Gene deletion
Single nucleotide polymorphism
Gene insertion
Horizontal gene tranfer (HGT)
Paired-end sequence reads
A
No coverage
G
Species A reference genome
Species B reference genome
B Individual-specific, temporally stable patterns in strain variation
Coverage at t=0
Coverage at t=1 year
Blue genes are stably present in individual 1 and stably absent in individual 2
Red genes are stably present in individual 2 and stably absent in individual 1
Coverage at t=0
Coverage at t=1 year
Genes from species A reference genome

Figure 5-11. Metagenomic profiling of strain-level variation in microbial communities. (A) Mapping paired-end sequencing reads to microbial reference genomes reveals not only the genomes that are present in a community, but also differences between the isolates of particular species and the reference isolate. In this example, most positions have 4x coverage, represented by four sequencing reads mapped to each position in the reference genome sequences from bacterial species A and B. Gene deletion events can be detected with relatively low coverage of the reference genome; no reads from the sample map to deleted genes (in orange). Higher sequencing coverage of the genomes facilitates differentiating between sequencing error and true nucleotide-level strain variation. Such variation includes fixed differences (in which the sample is consistently different from the reference at some site) and single nucleotide polymorphisms (SNPs; in which a site occurs in two or more states in the sample). Sequence reads that do not map together (blue reads from individual 1 and red reads from individual 2) indicate additional community variation, including the insertion of genomic material not found in the reference genome by mechanisms such as horizontal gene transfer (HGT). **(B)** Mapping reads to reference genomes can reveal patterns of gene presence or absence, which is a form of strain variation. Here, two individuals sampled at two time points (t = 0 and t = 1 year) are distinguished by the presence or absence of genes in species A. The blue genes are stably present in individual 1 and stably absent in individual 2, whereas the red genes are stably present in individual 2 and stably absent in individual 1. Adapted from Franzosa EA, Hsu T, Sirota-Madi A, Shafquat A, Abu-Ali G, Morgan XC, Huttenhower C. 2015. *Nat Rev Microbiol* **13(6):**360–372, with permission.

for establishing what constitutes a healthy microbiota so as to understand how changes in microbial composition, in terms of diversity and richness of content, affect health and disease. A large part of the HMP was to establish a repository of reference microbial genome sequences that could be used for facilitating the interrogation of microbiomes.

Metagenomic analysis starts with the census information indicating which species are present in microbial populations, but then adds information about metabolic function gleaned from thousands of complete bacterial genomes that are already deposited in genome databases, largely established through the HMP effort (see Box 5-1). These resources can provide a vast amount of information about the metabolic potential of a particular microbiome through comparison of the metabolic pathway sequences present in that mixed bacterial population with those from annotated genomes of taxonomically related bacteria that have been collected through the HMP. Since most of the already completed genomes were sequenced from a single clone of that microbe, most of the existing metabolic pathways and gene functions have already been ascertained, either directly from biochemical analyses or by inference based on analogy to similar genes in other microbes.

Beyond the Metagenome

A shared limitation of the 16S rRNA gene and metagenomic analysis approaches is that they do not provide information about which genes are being expressed at any given time or what the functional activity of the community is under any particular condition. Since many bacteria that normally reside in or on the body may be expelled into the environment after traveling through the stomach and intestinal tract from their site of origin, it stands to reason that the site they normally occupy does not explain the presence of all of their genes. They have to endure stresses independent of the environment of the site in which they usually reside. Thus, only a subset of genes is likely to be expressed at any one time or at any particular site. Moreover, even within the same site, changes in conditions, such as changes in diet or hormonal levels, may cause increases or decreases in expression of certain sets of genes. Work is now underway to include additional information about the functional status of the microbial community by combining other types of functional information about gene, RNA, and protein expression levels, activity status, and flux of metabolite content.

RNA-Seq Profiling (Transcriptomics). Gene expression in complex populations can be measured using techniques that detect and quantitate mRNA levels, including qPCR (see Figure 5-5) and **RNA-seq** technology (Figure 5-12). The Illumina RNA-seq paired-end sequencing platform has become the method of choice for interrogating the abundance and diversity of RNA transcripts (transcriptomics). The first step of RNA-seq is to extract and purify total RNA from the bacterial samples that will be compared. Different RNA purification methods are used for mRNA and small RNAs (sRNA) of less than 100 nucleotides. Highly abundant rRNAs and tRNAs are removed, and the mRNA samples are physically fragmented into smaller pieces. Reverse transcription is performed to convert the RNA fragments into cDNA, and the resulting cDNA is ligated to adapters, where barcode sequences mark cDNAs from different RNA samples. The cDNA + adapters are then subjected to the library amplification and Illumina sequencing, as illustrated in Figure 5-6.

The output of the Illumina sequencing is millions of short-sequence reads of about 150–300 nucleotides that correspond to regions of transcribed mRNA and sRNA molecules in the original samples. Multiple samples can be sequenced simultaneously in each Illumina sequencing run, and sequence reads can be sorted by the unique barcode sequences used in the adapters ligated to cDNAs of each sample. Because mRNA is randomly fragmented in this procedure, a series of overlapping reads covering the length of each mRNA emerges, and each nucleotide base in the mRNA is determined multiple times in separate reads. The total number of reads of each nucleotide base is referred to as coverage, and for most mRNA molecules the coverage of each nucleotide base is approximately equal. However, keep in mind that the output of a single RNA-seq experiment is all of the separate mRNAs or sRNAs expressed in a bacterium.

Because of the immense volume of data acquired, computer bioinformatic analyses are required to align and display the separate mRNAs and sRNAs from RNA-seq experiments. From the separate reads, three kinds of analyses are possible (Figure 5-12B). If a genomic DNA reference sequence is unknown, the overlapping reads can be aligned to indicate the length and sequence of individual mRNA molecules, which may contain open reading frames (Figure 5-12B, left). If a reference genomic DNA sequence is known, then the RNA-seq data can be compared to annotated reading frames and intercistronic regions to indicate operon arrangements (Figure 5-12B, middle). These kinds of analyses also provide identification of the locations of monocistronic and multicistronic operons, likely sRNA and regulatory RNAs that do not seem to encode proteins, and regions of antisense transcription (Figure 5-12C).

Probably the biggest application of RNA-seq is quantitation of the relative amounts of gene transcripts in a wild-type bacterium compared to that of a mutant or to that of the wild-type bacterium subjected to a stress condition (Figure 5-12B, right). The basis of this method is that the amount of cDNA synthesized and sequenced using the RNA-seq technology is proportional to the amount of mRNA or sRNA in the initial samples (Figure 5-12A). That is, the number of nucleotide base reads for each mRNA or sRNA is proportional to the amount of mRNA and sRNA in the samples. More reads across a gene or operon relative to a wild-type standard or to control conditions indicate an increased transcript amount, whereas fewer reads indicate less relative transcription.

In quantitation experiments, total RNA is extracted from the two strains whose transcriptomes are to be compared (e.g., a wild-type versus mutant strain), prepared for RNA-seq with two sets of barcodes, and then subjected to Illumina sequencing. Normalization between samples is performed by summing the number of reads for each gene and dividing by the gene length and the total number of reads in that sample. The relative change in transcript amount can then be calculated and compared for each gene in the bacterium. An example is shown in Figure 5-12D of the application of RNA-seq to determine the genes in *E. coli* whose relative transcript amounts increase or decrease in response to the glucose analogue, α-methylglucoside (αMG). As a final step in this transcriptome analysis, relative changes in transcript amounts detected by RNA-seq were confirmed by the independent method of quantitative reverse-transcription PCR (qRT-PCR). RNA-seq has largely replaced tiled microarrays for transcriptome analyses in bacteria and other organisms.

Coupling of RNA-seq results from total mRNA of a microbial community (**metatranscriptomics**) with the total genomic DNA content (metagenomics) enables an estimation of which metabolic pathways and protein functions are expressed in a particular sample under certain conditions. In this approach, relative mRNA transcript amounts are determined by the RNA-seq methods described previously. This

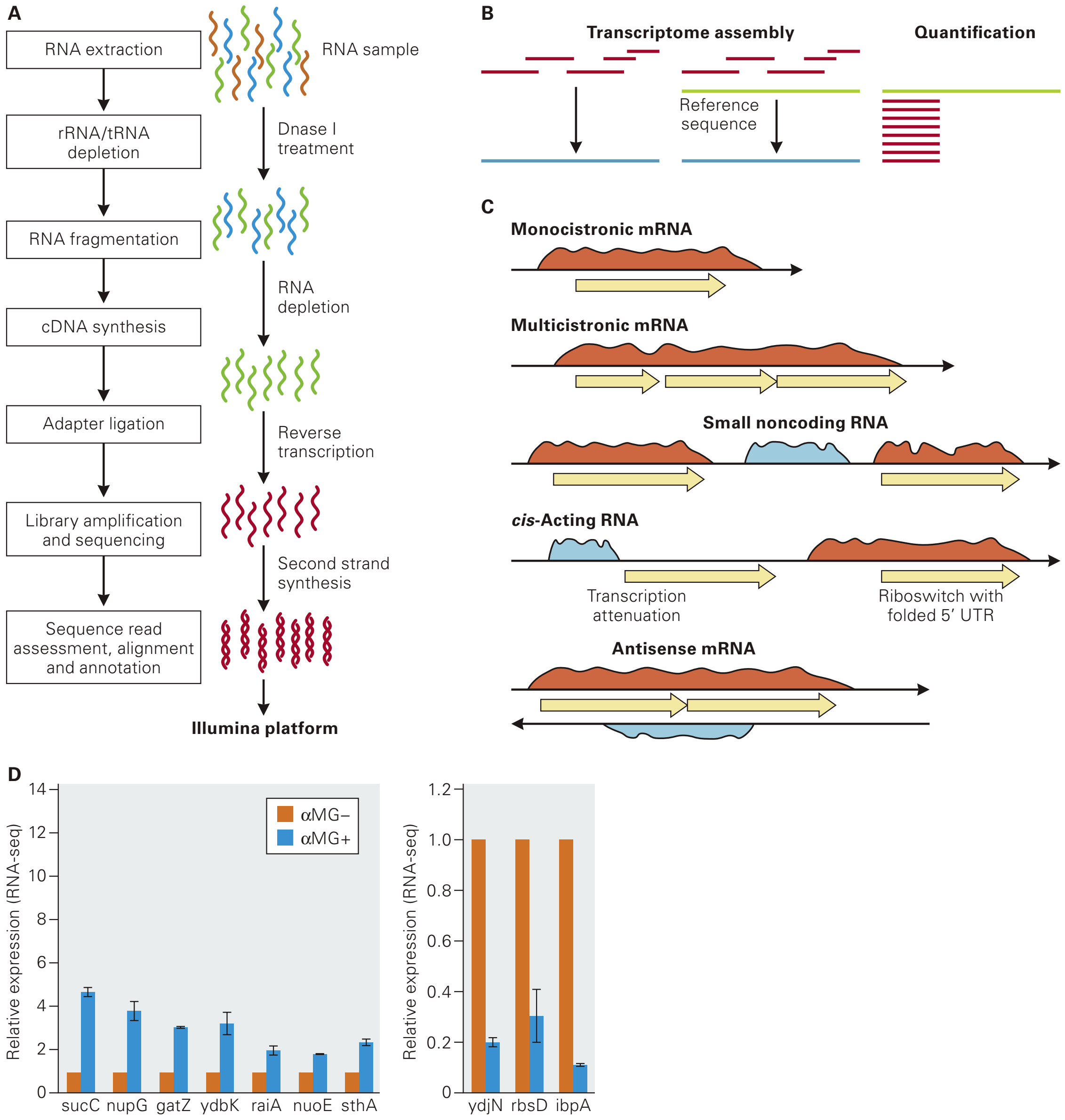
A
RNA extraction
RNA sample
rRNA/tRNA depletion
Dnase I treatment
RNA fragmentation
RNA depletion
cDNA synthesis
Adapter ligation
Reverse transcription
Library amplification and sequencing
Second strand synthesis
Sequence read assessment, alignment and annotation
Illumina platform
B
Transcriptome assembly
Quantification
Reference sequence
C
Monocistronic mRNA
Multicistronic mRNA
Small noncoding RNA
cis-Acting RNA
Transcription attenuation
Riboswitch with folded 5′ UTR
Antisense mRNA
D
Relative expression (RNA-seq)
αMG−
αMG+
0
2
4
6
8
10
12
14
sucC
nupG
gatZ
ydbK
raiA
nuoE
sthA
0
0.2
0.4
0.6
0.8
1.0
1.2
ydjN
rbsD
ibpA

Figure 5-12. RNA-seq technology. The Illumina RNA-seq platform enables global profiling of the transcriptional responses of all genes in individual cells or tissues at considerable depth of coverage under multiple conditions or over time. **(A)** Steps in an RNA-seq experiment using Illumina sequencing described in the text and Figure 5-6. **(B)** Outcomes of aligning multiple separate, short (150–300 nucleotides) sequencing reads, which correspond to overlapping segments of mRNA or sRNA. **Left**, if a reference genome is not available for a bacterium, the separate overlapping reads can be aligned to show the length of a transcript. The transcript can then be analyzed for open reading frames (ORFs) or other features. **Middle**, if a reference genome is available, the aligned fragments can be compared to genomic features, such as ORFs, intercistronic regions, or predicted promoters and transcription terminators. **Right**, the number of reads for each nucleotide base in a transcript is proportional to the starting amount of mRNA or sRNA present in the sample. Hence, following normalization, relative transcript amounts can be determined in different bacterial strains or samples, such as in a wild-type bacterial strain compared to a mutant strain or a wild-type strain grown under an unstressed versus a stressed condition. See the text for additional details. **(C)** The number of nucleotide base reads (blue and red regions) is often constant across regions that are cotranscribed. Therefore, the read patterns across genes indicate monocistronic versus multicistronic operons, and also the presence of any sRNA, putative regulatory RNAs, or antisense RNAs. See the text for additional details. **(D)** An example of changes in relative transcript amounts determined by RNA-seq. An *Escherichia coli* strain was grown in medium lacking or containing a glucose analogue, α-methylglucoside (αMG). RNA-seq analysis showed that the relative transcript amounts of seven metabolic genes increased (left) and three metabolic genes decreased (right) in the bacteria treated with αMG compared to the untreated control. Independent quantitative reverse-transcription polymerase chain reaction (qRT-PCR) experiments confirmed the trend in expression changes detected by RNA-seq. Panel D adapted from McClure R, Balasubramanian D, Sun Y, Bobrovskyy M, Sumby P, Genco CA, Vanderpool CK, Tjaden B. 2013. *Nucleic Acids Res* **41(14):**e140, with permission.

approach can provide an estimation of the metabolic potential and physiological makeup of that particular microbiota. For example, one study comparing the gut metagenome (i.e., the predicted metabolic genes present) with the corresponding metatranscriptome (i.e., the relative transcription levels of the metabolic genes present) revealed that certain pathways, such as sporulation and amino acid biosynthesis, were consistently under-expressed, whereas other pathways, such as ribosome biogenesis, stress response, and methanogenesis, were consistently overexpressed in relation to their DNA abundance. These findings are consistent with the presumed roles of these pathways under the conditions in the gut. For instance, you would expect an abundance of amino acids in the gut from the digestion of foodstuffs so that bacteria would not need to make their own, and thus amino acid biosynthetic genes would be downregulated. On the other hand, the rich nutrient environment of the gut would encourage bacterial growth, such that there would be a lot of protein synthesis activity and ribosomal subunit genes would be strongly upregulated.

Proteomics and Metabolomics—the Emergence of Multi-Omics. Even the advances in metagenomics and metatranscriptomics may not be enough to fully characterize the functional activity of microbial communities. Two genes in a microbial population may encode the same type of enzyme or transport protein. But different enzymes and different transport proteins have different affinities for their substrates and different levels of activity. Additionally, there may be regulatory factors that impinge on the observed functional activity. Thus, a gene that is expressed at a higher level than another gene may not be more important metabolically or physiologically. To address this need for functional understanding at a population level, other measures of functional potential or activity of the microbiota are necessary.

Measuring the relative abundance of protein and metabolite (small molecule) amounts provides another indicator of the functional activity of a microbial community. Two approaches are now taking off that enable the assessment of proteins and metabolites in biological samples. Proteomics allows assessment of relative protein amounts and stabilities and functional states (activation, inactivation, or modification) of the protein content of samples (Figure 5-13). Metabolomics allows the determination of the composition of all of the metabolites and other small molecules (usually any molecule with a molecular mass less than 1,500 Da) present in a sample (Figure 5-14). Both approaches used to determine protein or metabolite content and abundance depend on chromatographic separation methods, usually **high-performance liquid chromatography (HPLC)** or **gas chromatography (GC)**, followed by detection, structural determination, identification, and quantification of the contents using analytical techniques, such as **mass spectrometry (MS)**, **nuclear magnetic resonance (NMR) spectroscopy**, or some other type of spectroscopy.

Currently, comprehensive proteomic and metabolic profiling is sometimes a daunting undertaking, even in monocultures of one kind of bacterium. Each of the previously mentioned approaches add an extra level of technological and computational scale and complexity to the profile analyses, which make applications toward characterization of populations of bacteria in the microbiota very challenging. For example, while proteomics is one of the fastest growing areas of research today, the sheer number of protein variants possible for each gene product and its homologs in many bacteria from multiple phyla places a considerable burden on the bioinformatics required to appropriately annotate and classify the proteins evolutionarily based on sequence similarity.

Proteomic analysis is highly valuable in establishing whether a protein encoded by a gene is expressed at any given time. More sophisticated proteomic approaches (Figure 5-13) can determine relative amounts of proteins in a bacterium or population; therefore, proteomics can provide information regarding whether certain proteins have the potential to be functionally active in cells. However, this does not necessarily mean that the biochemical and cellular function of different variants have been established to be the same in all bacteria that harbor a homolog of a given gene, nor does it definitively establish the function of the expressed protein or proteins encoded by that gene. Moreover, the presence of a protein does not necessarily mean that it is functionally active, since it does not provide any information about whether its activity has been modulated through interactions with or modifications by other proteins or ligands in regulatory signaling networks or biosynthetic pathways.

Metabolomics also presents experimental challenges. It is not possible with today's technologies to analyze all of the metabolites in a given sample by any single analytical method. In any given biological sample there are a wide range of primary and secondary metabolites involved in essential and nonessential metabolic and signaling pathways, including peptides, oligonucleotides, sugars, nucleic acids, ketones, aldehydes, alcohols, amino acids, amines, lipids, steroids, alkaloids, and other endogenously generated small molecules. In addition, numerous xenobiotics, such as drugs and antibiotics, are also often present in varying amounts in microbiota samples. All of these molecules have very different chemical and structural properties, including different functional features, hydrophobicity, acidity, redox potential, and reactivity. Consequently, multiple and different separation and detection methods must be applied in combination to comprehensively identify and quantify the enormously diverse metabolites in a single bacterium or combinations of bacteria.

Moving to the next level of complexity, a few intrepid researchers have begun to explore the application of these ge-

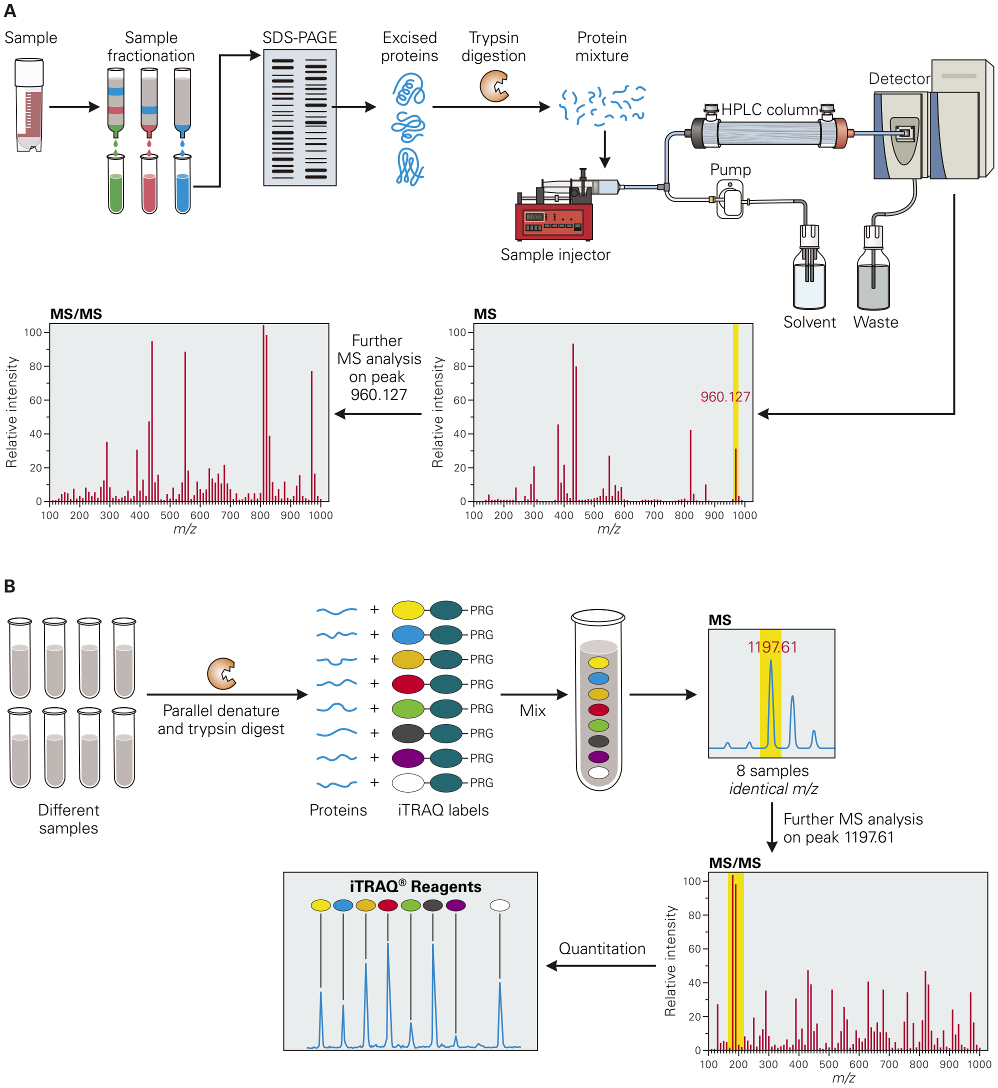
A
Sample
Sample fractionation
SDS-PAGE
Excised proteins
Trypsin digestion
Protein mixture
Detector
HPLC column
Pump
Sample injector
Solvent
Waste
MS/MS
Relative intensity
m/z
Further MS analysis on peak 960.127
MS
960.127
B
Different samples
Parallel denature and trypsin digest
Proteins
iTRAQ labels
PRG
Mix
MS
1197.61
8 samples identical m/z
Further MS analysis on peak 1197.61
MS/MS
Quantitation
iTRAQ® Reagents

Figure 5-13. Overview of proteomics technology. Proteomics provides a comprehensive assessment of the protein identity and relative abundance in biological samples. **(A)** In the simplest identification, proteins in extracts of a bacterial culture are first fractionated by liquid chromatography (LC) and SDS polyacrylamide gel electrophoresis. Individual bands are cut out from gels, and the proteins are digested into peptides with trypsin protease. The resulting peptides are fractionated by high-performance liquid chromatography (HPLC) that is connected in-line to a mass spectrometer detector. The mass/charge (m/z) ratio is then determined for the peptides in the HPLC fraction that can be detected by mass spectrometry. Computer programs are then used to generate *in silico* predictions of the m/z ratios of all of the tryptic peptides that correspond to proteins predicted from the bacterial genome. The computer then matches the predictions with the peptide profile to identify the unknown protein. The identity of tryptic peptides can be further confirmed by tandem mass spectrometry (MS-MS) analysis, where fragments of the peptides to allow further structural determination of the peptides. With the newest ultra-performance LC systems and mass spectrometer detectors, this analysis has been expanded to complex mixtures of proteins in crude extracts. The crude protein mixtures are digested by trypsin and analyzed by liquid chromatography-mass spectrometry. The presence of as many as 700 proteins can be demonstrated by this method. However, these methods are only semiquantitative for determining the relative abundance of proteins in samples. Nevertheless, protein interaction or regulatory networks can be built for various purposes, such as signaling pathways, biosynthetic pathways, or protein-protein interaction pathways from these proteomic approaches. **(B)** iTRAQ method to quantitate relative protein amounts in different samples. As with RNA-seq, a major goal of proteomics is often to determine how the relative amounts of proteins change during bacterial growth in a standard unstressed condition compared with stressed conditions or in a wild-type compared to a mutant strain. An ingenious method, called iTRAQ, has been developed for this purpose. In an iTRAQ experiment, protein profiles from as many as eight separate cultures or conditions can be compared. The separate extracts are denatured and then digested to completion with trypsin. Each extract is reacted with one of the eight available iTRAQ labels, which do not change the relative m/z ratio for any given tryptic peptide. However, when each iTRAQ-labeled peptide is fragmented by MS-2, the peptides from the different samples end up with slightly different m/z values that allow resolution and quantitation of the iTRAQ-labeled fragments. The relative amount of each of the different iTRAQ-labeled peptide fragments is directly proportional to the amount of each protein that contains the peptide in the original samples. Adapted from PTM Biolabs (http://ptm-biolab.com/itraq-proteomics), with permission.

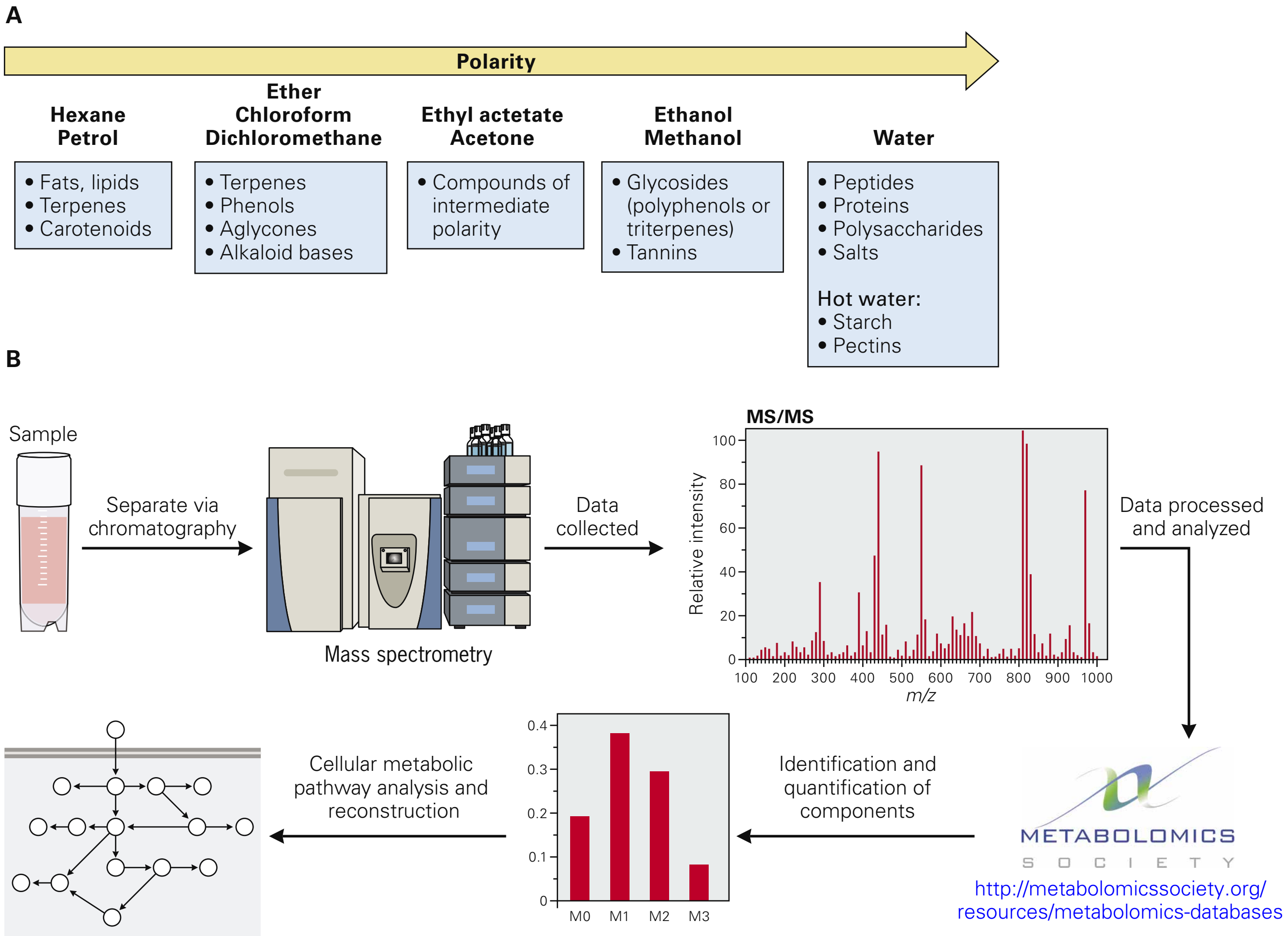
A
Polarity
Hexane
Petrol
• Fats, lipids
• Terpenes
• Carotenoids
Ether
Chloroform
Dichloromethane
• Terpenes
• Phenols
• Aglycones
• Alkaloid bases
Ethyl actetate
Acetone
• Compounds of intermediate polarity
Ethanol
Methanol
• Glycosides (polyphenols or triterpenes)
• Tannins
Water
• Peptides
• Proteins
• Polysaccharides
• Salts
Hot water:
• Starch
• Pectins
B
Sample
Separate via chromatography
Mass spectrometry
Data collected
MS/MS
Relative intensity
100
80
60
40
20
0
100
200
300
400
500
600
700
800
900
1000
m/z
Data processed and analyzed
METABOLOMICS
SOCIETY
http://metabolomicssociety.org/resources/metabolomics-databases
Identification and quantification of components
0.4
0.3
0.2
0.1
0
M0
M1
M2
M3
Cellular metabolic pathway analysis and reconstruction

Figure 5-14. Overview of metabolomics technology. Metabolomics technology enables a comprehensive analysis of the content and relative abundance of low molecular weight (5–1,500 Da) molecules or metabolites within a sample, which provides a better assessment of which metabolic pathways are active and what the relative flux through those pathways is. **(A)** The samples are processed using solvent extraction methods with various polarities that are specific for a particular type of molecule, such as lipids, sugars, or small organic molecules. **(B)** The extracted compounds are then separated from each other via chromatographic methods such as high-performance liquid chromatography or gas chromatography either directly or after chemical derivatization (to enhance solubility). This is followed by structural analysis using detection technologies, such as mass spectrometry or nuclear magnetic resonance spectroscopy. The data are then processed and analyzed using bioinformatics tools and comparison with existing data from known compounds in available databases to determine the structure and identity of the components and their relative abundance in the sample. The metabolites identified are then mapped onto cellular signaling and biosynthetic pathways. A comprehensive list of metabolomics databases and resources can be found at the website for the Metabolomics Society (http://metabolomicssociety.org).

nomic and functional approaches toward the characterization of microbiota within the context of the host response and the interplay of host-microbe interactions. Importantly, an integrated multi-omics approach is critical for interpretation of the data obtained from each of the separate approaches to gain deeper biological insights. For instance, inclusion of gene copy number in a sample (obtained from genomic data) can be used to normalize the functional activity observed for a particular set of expressed genes with similar enzyme activities (obtained from transcriptomic, proteomic, or metabolomic data). A multi-omics approach also allows the researcher to confirm conclusions or hypotheses made from one set of data, such as the presence of a gene that has weak homology to a known gene in a known biosynthetic pathway that makes a particular metabolite (obtained from comparison of metagenomic data with databases) with correlative functional data obtained through other methods, such as the presence of the expressed protein (obtained from proteomic data) and/or the presence of the predicted metabolite (obtained from metabolomic data). Again, a comprehensive discussion of this complex topic is beyond the scope of this textbook, but we provide a few examples later and in the selected readings for those who are curious.

Overview of the Human Microbiota

A human fetus is devoid of microorganisms. Passage through the vaginal tract during birth begins the colonization process. Shortly after birth, the microbiota profiles of vaginally delivered infants resemble that of their mother's vagina, while the microbiota profiles of infants delivered via Cesarean section more closely resemble that found on the mother's skin (Figure 5-15). This process is further influenced by exposure to early environmental factors and continues as the infant grows. The final microbiota composition is not achieved until the child is about 2 or 3 years old. Once the microbiota of an area assumes its stabilized, steady-state form, different areas of the body harbor very different microbial populations. Even within a single site, such as the mouth, different areas may contain different sets of microbes (Figure 5-16). This diversity is not surprising in view of differences between conditions that microbes encounter in any particular site. Nevertheless, despite these site-to-site differences within an individual, microbial community compositions at particular sites are generally more similar among cohabiting family members, less similar among distant relatives, and vary considerably among unrelated individuals.

There are, however, some features of the microbiota that are common to all sites of the human body colonized by microbes. First, the numerically predominant microbes are bacteria. Archaea, fungi, and viruses (including bacteriophage) are also present, but archaea and fungi are in much lower numbers. Second, the majority of the bacteria

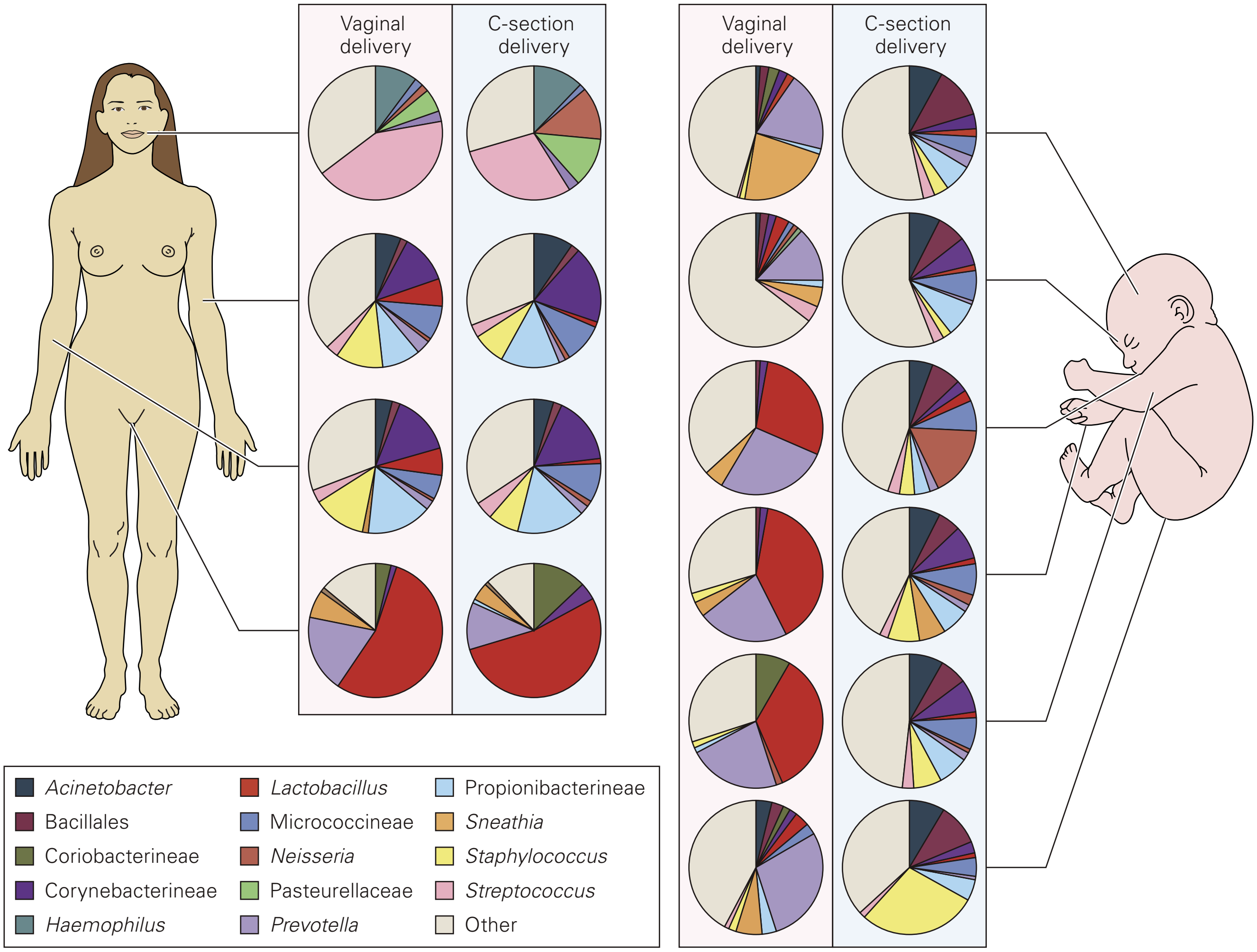

Figure 5-15. Birth delivery mode influences the microbiomes of newborn infants. Shown are pie charts of the genus-level bacterial compositions derived from 16S rRNA gene sequence analysis at different body sites for a group of mothers and their babies shortly after birth, grouped according to the delivery method of the babies: vaginal versus Cesarean section (C-section). Adapted from Reid G, Younes JA, Van der Mei HC, Gloor GB, Knight R, Busscher HJ. 2011. *Nature Rev Microbiol* **9(1)**:27–38, with permission; based on data from Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. 2010. *Proc Natl Acad Sci USA* **107(26)**:11971–11975.

are Gram-positive bacteria. In fact, some of the microbial population shifts that are associated with such conditions as periodontal disease and BV involve a change from a predominantly Gram-positive to a predominantly Gram-negative bacterial population. Why the Gram-positive bacteria dominate the human microbiota is not clear. Finally, although the microbiota of different areas of the body is usually protective, many members of the microbiota can cause serious infections, even in healthy individuals, if they manage to breach the protective epithelial or mucosal layers and enter normally sterile areas of the body such as blood and tissue.

Skin Microbiota

The surface of the skin is a dry, slightly acidic, and aerobic environment. Early culture-based studies determined that Gram-positive staphylococci, such as *Staphylococcus epidermidis,* are the predominant bacteria occupying this site, although it is not uncommon to have transient colonization of the skin by soil bacteria or bacteria from other parts of the body. Skin bacteria like *S. epidermidis* were long assumed to be unable to cause disease. Finding them in a blood specimen, for example, was considered to be proof that it was introduced via a careless health care worker or a technician that had contaminated the specimen. Although the surface of the skin is aerobic, pores and hair follicles can be anoxic enough to support the growth of anaerobic bacteria, such as *Propionibacterium acnes* (see chapter 2). Today's view of the skin microbiota is completely different. Comparison of 16S rRNA gene-based community profiles from skin at different locations showed not only high variability from person to person (interpersonal variation), but also high variability within an individual (intrapersonal variation) (Figure 5-16). While Propionibacteriaceae dominate the microbiomes of head regions (60–80%) and arms (20–40%), the torso and legs are dominated by *Staphylococcus* and *Corynebacterium* species. Skin sites also vary considerably in the bacterial diversity of minor bacterial species present. Indeed, it has been proposed that skin bacterial community profiles could serve as a valuable aid in forensic identification.

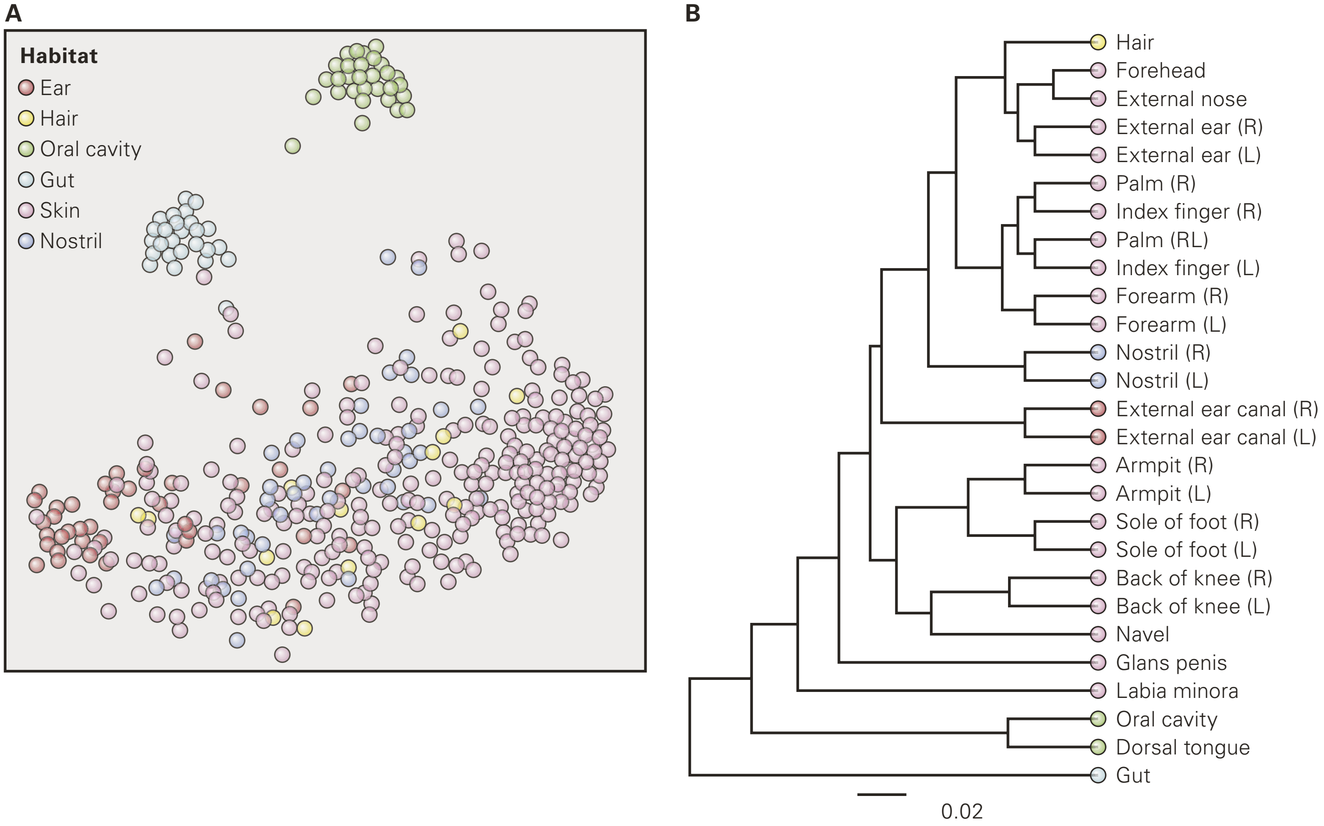

Figure 5-16. Phylogenetic relationships of microbiome profiles among human body sites. Phylogenetic comparison of 16S rRNA gene-based bacterial community profiles among different human body sites revealed strong clustering by body site, meaning community compositions varied significantly less within a particular body site than between sites. Each point in the principal-component analysis (PCA) plot in **(A)** and the dendrogram (tree) in **(B)** corresponds to the profile of a sample, colored according to particular body site. In these clustering analyses, 16S rRNA gene-based bacterial community profiles that cluster closer together are more similar to each other. Adapted from Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R. 2009. *Science* **326(5960):**1694–1697, with permission.

Oropharyngeal Microbiota

The most common source of *S. aureus*, a major cause of hospital-acquired and community-acquired infections, is the human nose, where it is most abundant in the upper part of the nasal cavity. At any particular time, about one-third of the human population harbors *S. aureus.* The nasopharynx is also home to *S. pneumoniae*, another human commensal bacterium that can cause a variety of serious invasive diseases elsewhere in the body. About 25% and 40% of healthy adults and children, respectively, carry *S. pneumoniae* at any given time. Lower in the nose, not surprisingly, are bacteria that are also found on the skin. Several large-scale efforts are currently underway to understand the composition of the microbiota and the dynamics of colonization of the nose.

The microbiota of the mouth and throat is fairly well characterized, largely due to the involvement of the oral microbiota in periodontal disease, a major cause of gum disease and tooth loss in adults. The microbiota of the healthy mouth consists largely of facultative Gram-positive bacteria, mainly streptococci such as *Streptococcus mutans* and *Streptococcus salivarius,* which are not completely innocuous. These bacteria ferment sucrose to lactic acid, which in turn contributes to the development of dental caries. Utilization of sucrose also results in production of the polysaccharide dextran, which binds bacteria together and allows plaque to form. In periodontal disease, this Gram-positive microbiota shifts to a Gram-negative anaerobic microbiota in the area of the gums. The space between the gums and the upper portion of the tooth surface is called the periodontal pocket. It is a fairly anoxic area and so is able to support the growth of obligate anaerobes, such as *Porphyromonas gingivalis, Prevotella* spp, *Tannerella forsythia*, and *Treponema denticola*. These species produce proteases and other tissue-degrading enzymes, and this may be a major cause of the inflammation that characterizes the disease. As we will discuss at length in chapter 6, *P. gingivalis* appears to act as a "keystone" or "trigger" pathogen by altering the normally benign composition of the commensal microbiota, leading to microbial dysbiosis and increased inflammation and periodontal disease.

More recently, scientists have found that bacteria involved in periodontal disease, such as the Gram-negative anaerobe *Fusobacterium nucleatum*, are responsible for some cases of preterm birth. The hypothesis is that the bacteria enter the bloodstream through the inflamed gum tissue and lodge in the placenta. The resulting inflammation causes the fetus to be delivered prematurely. Similarly, others have suggested that oral bacteria associated with gingivitis might enter the bloodstream, causing inflammation in the blood vessels and thus leading to heart disease. In fact, it is now established that some oral bacteria, such as *Streptococcus sanguinis* and *Streptococcus pyogenes*, are direct causes of endocarditis. These examples illustrate the new thinking about connections between alterations in the normal microbiota and the impact on diseases in other areas of the body. More examples can be found in the suggested readings at the end of this chapter.

Microbiota of the Small Intestine and Colon

The small intestine is characterized by the fast flow of contents (see chapter 2). The fast flow helps wash bacteria out of the site. Throughout most of the small intestine, fast flow of contents prevents bacteria from adhering to the mucosa in order to stay in the site. The human microbiota of the small intestine is poorly characterized, largely because it is difficult to obtain samples from that area. Samples are usually obtained through a swallowed tube that works its way into the small intestine. Although such tubes have been used, from a microbiological perspective they have a significant deficiency; namely, they sample the lumenal but not the adherent microbiota. In mice, where such studies have been feasible, there is an adherent microbiota of the small intestine that consists mostly of *Clostridium* species.

The lower intestine or colon, by contrast, has a much slower flow of contents (see chapter 2) and much higher concentrations of adhering and colonizing bacteria (Figure 5-17). Bacteria make up a third of the microbial content of the human colon. These microbes have a complex relationship with us. We provide them with ingested food and fermentable substances like mucins, while the bacteria further degrade complex, undigested foodstuffs into substances that contribute to our nutrition. The human small intestine can absorb small molecules such as mono- and disaccharides, and can digest soluble starch, but most of the complex polysaccharides in the human diet, such as cellulose, xylan, and less soluble starch, pass through to the colon. Colonic bacteria ferment carbohydrates, including these polysaccharides, to produce CO_2, H_2, and short-chain fatty acids (SCFAs; acetate, propionate, and butyrate), which are absorbed by intestinal cells and used as a source of carbon and energy, providing as much as 8–10% of human nutrition. Colonic bacteria also ferment host-produced polysaccharides, such as mucopolysaccharides and mucins.

Colonic bacteria not only break down food material into simple sugars, amino acids, and lipids (**catabolism**), but also synthesize other nutrients and vitamins (**anabolism**) that our bodies need. Indeed, emerging experimental evidence points to a number of important functional roles that microbiota play in providing metabolites and host-microbe interactions that stimulate multiple endocrine, neurocrine, and immunologic signals to the brain and vice versa. These bidirectional interactions have been dubbed the **microbiota-gut-brain axis**. Recent studies have shown that modulating the gut microbiota impacts emotional behavior, including anxiety, depression, and pain, supporting the therapeutic potential of appropriate probiotics, prebiotics, and diet as interventions for psychiatric and neuroimmune disorders.

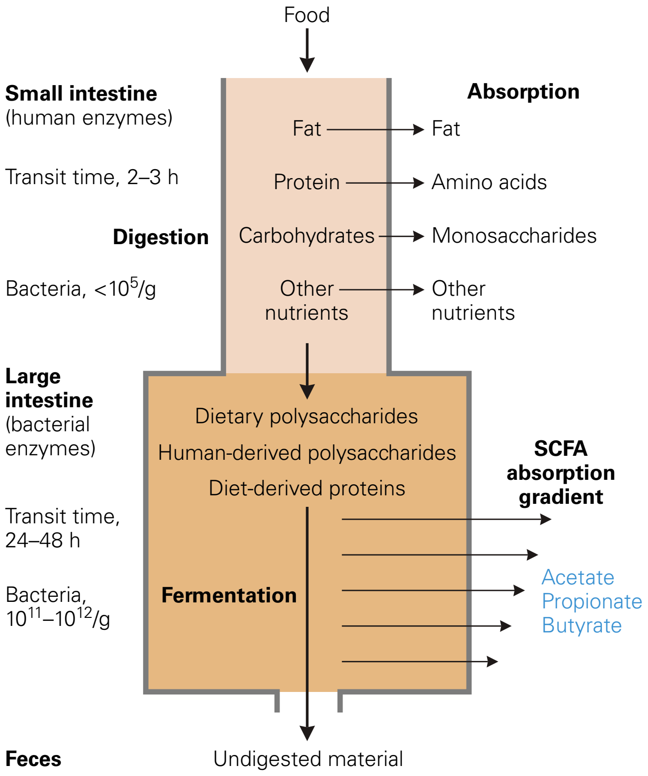

Figure 5-17. A schematic view of activities of the colonic microbiota. In the small intestine, concentrations of bacteria are low due to the fast flow of contents and human intestinal enzymes that mediate most of the digestion. In the colon, concentrations of bacteria are so high that bacteria account for about 30% of the volume or contents. In this site, the colonic bacteria ferment polysaccharides from the human diet (plant polysaccharides or dietary fiber), host-derived polysaccharides (mucins and mucopolysaccharides), and diet-derived proteins (those not digested by pancreatic enzymes in the small intestine). The resulting short-chain fatty acids (SCFAs; 85–95% of SCFAs produced in the colon are acetic acid, propionic acid, and butyric acid) and amino acids are absorbed by the human body and used as sources of carbon and energy.

The colon is an anoxic environment, so it is not surprising that the numerically predominant colonic bacteria are obligate anaerobes. The numerically predominant anaerobes include the phyla Bacteroidetes (Gram-negative *Bacteroides* species), Firmicutes (Gram-positive bacteria, including *Lactobacillus* and *Clostridium* species), and Actinobacteria (*Corynebacterium* and *Mycobacterium* species). Facultative bacteria such as *E. coli* and *Enterococcus* species are also present in lower numbers. *Bacteroides* species are the ones that are thought to play a major role in fermenting the dietary polysaccharides that our bodies cannot digest. Sequence analyses of the gut bacterial communities have shown that obese individuals have fewer Bacteroidetes (5%) and more Firmicutes (85%) than lean individuals (25% Bacteroidetes, 75% Firmicutes). Understanding colonic fermentation, together with 16S rRNA gene analyses of the microbiota of the colons of obese humans or mice compared to those of non-obese individuals, has fueled the notion that obesity might be caused in part by the composition of the colonic microbiota (Box 5-2), which could be treated through manipulation of the microbial contents of the gut.

The products of fermentation (acetate, CO_2, and H_2) are also used as carbon and energy sources by minor populations of methanogenic archaea. These methane producers convert H_2 and CO_2 to methane, which is absorbed, though not used, by the human body and is expelled in breath and flatus. About one-fifth of people tested have enough methane produced in their colons to be easily detectable in their breath by gas-liquid chromatography. Sulfate-reducing bacteria also reside in the colon, using H_2 to help convert sulfate to HS. The main source of the sulfate that they reduce is not clear, but some of it probably comes as a byproduct of fermentation of human-produced sulfated glycoproteins and sulfated polysaccharides, such as mucopolysaccharides and mucin. Sulfides produced by the sulfate reducers are responsible in part for the odor of feces and are thought to contribute as a risk factor for colon cancer.

In judging the energy balance, it is worth realizing that intestinal microbes also take a toll from us; they stimulate the immune system and the turnover of intestinal mucosal cells. The constant sloughing of intestinal mucosal cells is a very effective defense that prevents bacteria that have attached to the mucosal cells from staying in the site long enough to invade. Similarly, the intestinal immune system is an important defense. But these activities require an output of proteins and energy by the human body. On balance, however, the energy balance seems to go in our favor, unless you count the effort we put into cultivating and obtaining the foods we eat that ultimately feed the colonic bacteria.

Many colonic bacteria, such as *Bacteroides* species and numerically minor populations like *E. coli, Enterococcus,* and some *Clostridium* species, are capable of causing serious infections if they escape from the colon as a result of surgery or some other trauma and get into the bloodstream and tissues. How could obligate anaerobes like *Bacteroides* cause infection in the human body, which would seem to be an aerobic environment that could kill them? *Bacteroides* prefer to lodge in regions of prior tissue damage. Disruption of the blood supply to such areas causes them to become anoxic, and thus fertile ground for an anaerobic infection. Moreover, blood itself is actually a hypoxic environment that is low in free oxygen. These normal colonic inhabitants are increasingly becoming resistant to many antibiotics.

Bacteria in the intestine interact with each other metabolically in the sense that methanogens and sulfate reducers use the end products of the polysaccharide fermenters, but they also interact with each other genetically by exchanging DNA. There is an old idea called the reservoir hypothesis that frames this interaction in terms of the transfer

Box 5-2.
We Are What We Eat, or Rather What Our Microbiota Eats

Conventional wisdom has it that obesity is a result of genetics, lack of exercise, or a poor diet. But what if your intestinal bacterial population also contributes? An early study that used 16S rRNA gene profiling found that the microbiota of obese mice and humans differed from that of lean mice and humans. Moreover, when germfree mice (mice lacking any intestinal bacteria) were colonized with an "obese microbiota," those mice gained more fat than germfree mice colonized with a "lean microbiota." The main difference was the ratio of the two numerically predominant bacteria, the Bacteroidetes (*Bacteroides* species) and the Firmicutes (Gram-positive obligate anaerobes), in which a higher proportion of Bacteroidetes was associated with leanness.

The hypothesis that the composition of the colonic microbiota is associated with obesity is, as you might imagine, quite controversial, especially among those committed to theories that give precedence to exercise or diet. Also, association of obesity with a more active colonic fermentation seems to run counter to the belief that high-fiber diets would be associated with increased colonic fermentation due to the fact that fiber is primarily composed of polysaccharides that are fermentable by colonic bacteria. The efficiency of the fermentation may be a factor. If so, the prediction from the obesity studies would be that the Firmicutes are more efficient fermenters than the Bacteroidetes. Since virtually nothing is known about the Gram-positive anaerobes and their carbon sources, this is difficult to assess. Another possibility is that some fermenters take a lower energy toll in the form of stimulating mucosal cell turnover.

A good feature of the hypothesis regarding a connection between obesity and the microbiota composition is that it may prompt more studies of the metabolic activities of Gram-positive anaerobes. Moreover, it illustrates the fact that the 16S rRNA gene approach, and even the metagenomics approach, may be a good start for addressing these questions, but that work on better understanding bacterial physiology will be critical.

The idea that we might be able to combat obesity by manipulating our microbiota has received some strong experimental support. To study the impact of microbiota composition on obesity, germfree mice were inoculated with microbiota from obese or lean human twins fed a low-fat, high-fiber diet. As illustrated in panel A of the figure mice that received a microbiota from the obese twin (red) became obese, while mice that received a microbiota from the lean twin (blue) did not become obese. When the two groups of mice were housed together and fed a low-fat, high-fiber diet (as illustrated in panel B of the figure), transmission of microbes (mostly *Bacteroides* species) from the mice with a lean-promoting microbiota occurred to the mice with an obesity-promoting microbiota, such that none of the mice became obese. On the other hand, when the two groups were fed a high-fat, low-fiber diet, no transmission occurred and the mice with an obesity-promoting microbiota became obese.

Sources:

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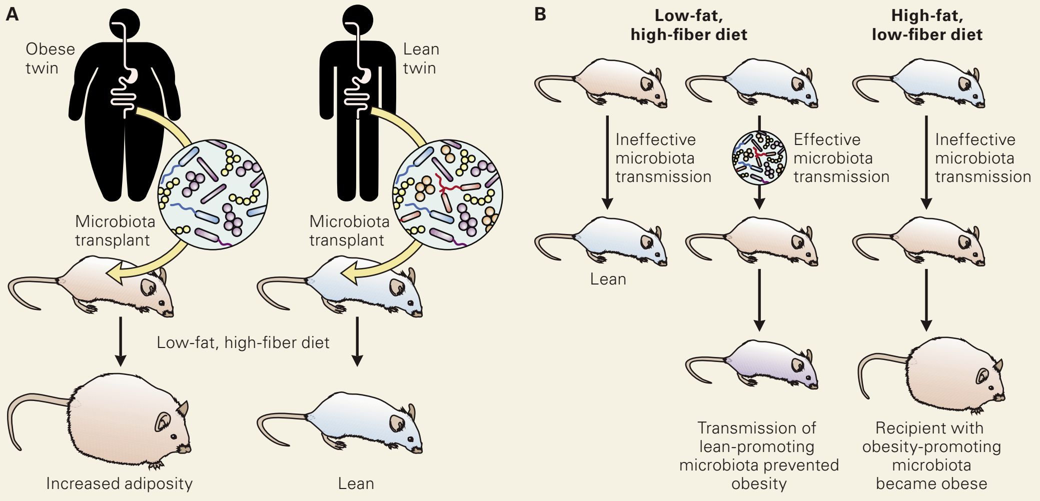
A
Obese twin
Lean twin
Microbiota transplant
Microbiota transplant
Low-fat, high-fiber diet
Increased adiposity
Lean
B
Low-fat, high-fiber diet
High-fat, low-fiber diet
Ineffective microbiota transmission
Effective microbiota transmission
Ineffective microbiota transmission
Lean
Transmission of lean-promoting microbiota prevented obesity
Recipient with obesity-promoting microbiota became obese

of antibiotic resistance and virulence genes (more on this in chapter 7). Briefly, colonic bacteria exchange DNA with each other. They may also exchange DNA with swallowed bacteria that are present only transiently in the colon as they pass through and are expelled into the environment. In this view, colonic bacteria act as reservoirs of antibiotic resistance and virulence genes in the sense that they are present in high numbers in an area in which other bacteria are transiently present for 24–48 hours, which is more than enough time for DNA transfers to occur. Only recently has it been possible to test this hypothesis by using molecular sequencing methods to follow the host-to-host transmission and movement of particular genes through and within the human colon. In fact, evidence is mounting that gene transfers via conjugative elements like plasmids and conjugative transposons occur frequently in the colon. These transfers occur between different species and genera, including between colonic bacteria and bacteria from different sites.

The reservoir hypothesis has loomed large in the debate over possible adverse consequences of the overuse of antibiotics, particularly on the farm. Antibiotic-resistant bacteria arise on the farm due to selection by the use of antibiotics as feed additives or to prevent infections in crowded populations of animals. The problem is that these antibiotic-resistant bacteria are already moving through the food supply and into the human intestinal tract, where the resistance genes could be transferred to bacteria permanently or temporarily residing in the human colon. These concerns regarding the increased spread of antibiotic resistance are compounded by the recent realization of the importance of a normal microbiota in maintaining health and by the fact that antibiotic treatment leads to loss of not just the disease-causing pathogen, but also the beneficial bacteria that maintain a healthy microbiota. Perturbation of the normal healthy microbiota (**dysbiosis**) has been correlated with inflammatory and autoimmune diseases, such as **inflammatory bowel disease (IBD)** and **Crohn's disease**, and with increased susceptibility to other types of infections by opportunistic pathogens such as *C. difficile*.

Microbiota of the Vaginal Tract

The microbiota of the female vaginal tract has already been introduced in the example about the application of DNA-based analysis of complex microbial populations, but that description did not explore the special features of the site that presumably explain the composition of the microbiota of the vagina. The vagina is a complex site. The vaginal tract experiences considerable periodic hormonal changes associated with the menstrual cycle. Although mucin secretions are constantly bathing the vaginal mucosa, the flow of fluids seen is not nearly as fast as that seen in the intestinal tract, except during menstruation. Most of the time, the bacteria are loosely or strongly associated with the vaginal mucosa.

The microbiota of the vagina of most healthy humans consists mainly of Gram-positive *Lactobacillus* species. These lactobacilli are fermentative bacteria that produce mainly lactic acid and probably contribute to the normally low pH (less than 5) of the vaginal tract, which generally acts as a powerful protective barrier against colonization by many disease-causing bacteria. Unfortunately, many other pathogenic bacteria can survive and multiply at pH 5. Another possible contribution of lactobacilli to protecting the vaginal tract against disease-causing bacteria is that some of them produce hydrogen peroxide, which is toxic to many microbes, not just bacteria. There is no question that women who take antibiotics that kill or inhibit the growth of lactobacilli often develop yeast infections (**vaginitis**) or bacterial dysbiosis (**vaginosis**). The availability of new molecular techniques has now allowed in-depth analysis of the vaginal microbiota and interrogation of their roles in vaginal health and reproduction.

BV was once considered to be a minor disease, characterized by slight discomfort, mild inflammation, a fishy odor, and an unhealthy vaginal microbiota that lacked lactobacilli. More recently, BV has been shown to be associated with a number of disorders, including increased risk of premature labor and birth, although the mechanism of this connection is still unclear, as well as increased susceptibility to infection with sexually transmitted pathogens, such as HIV, *Neisseria gonorrhoeae,* or *Chlamydia trachomatis*.

In contrast to diseases caused by a single pathogen, BV arises from a shift in the microbiota from a predominantly lactobacilli population to a predominantly Gram-negative one, principally *G. vaginalis*. In fact, a woman with a significant concentration of Gram-negative bacteria like *G. vaginalis* had previously been assumed by physicians to be exhibiting disease. As mentioned before, DNA-based analyses of a large number of apparently healthy women revealed a surprising finding: many of them carried high concentrations of *G. vaginalis* (see Figure 5-18). And, interestingly, while about 70% of the healthy women had predominantly lactobacilli, about 30% had very few or no lactobacilli.

More detailed metagenomic and multi-omic analyses have already demonstrated not just considerable person-to-person variation but variations within a person over the course of the menstrual cycle (Figure 5-18), as well as among different sites in the vaginal tract and with other factors such as hormonal status, sexual activity, age, and pregnancy. Clearly, the microbiota of the vaginal tract is turning out to have as complex a population as that of the other body sites. And, once again, an in-depth discussion of these fascinating topics is beyond the scope of this textbook, so we have included a few suggested readings at the end of this chapter.

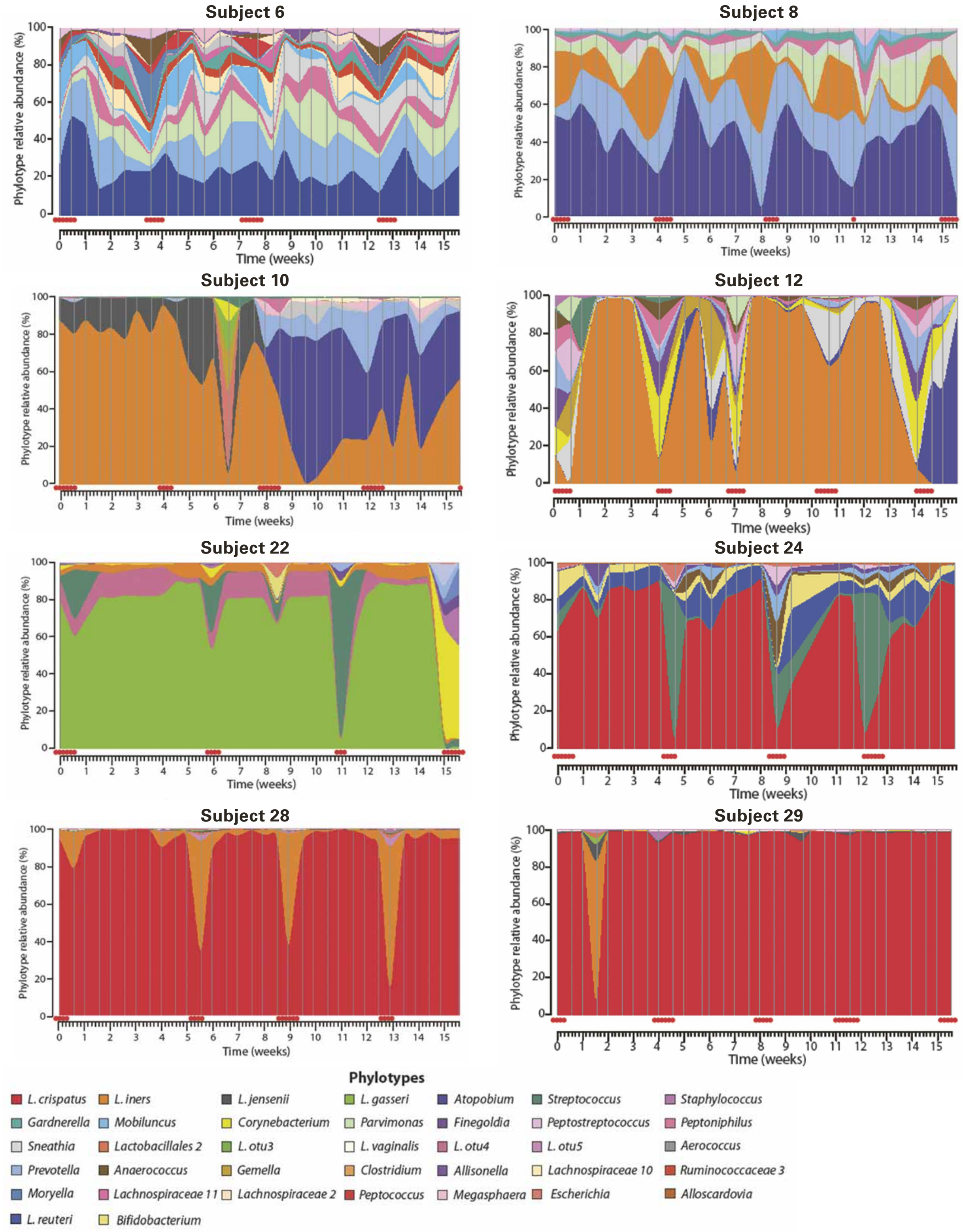

Figure 5-18. Temporal and interindividual dynamics of vaginal bacterial communities. Shown are the 16S rRNA gene-based bacterial community composition profiles of eight different healthy women sampled twice weekly over the course of 16 weeks. Colors indicate the relative abundance of each taxonomical group (phylotype) present in each sample. Red bars along the bottom of the plot indicate dates of menstruation. Reprinted from Gajer P, Brotman RM, Bai G, Sakamoto J, Schütte UM, Zhong X, Koenig SS, Fu L, Ma ZS, Zhou X, Abdo Z, Forney LJ, Ravel J. 2012. *Sci Transl Med* **4(132):**132ra52, with permission.

The Other Microbiota: The Forgotten Eukaryotes

The content of this chapter has so far focused largely on bacteria (with a brief foray into the archaea of the gut), so it is appropriate to end with a brief description of microbes that have been largely ignored in most studies, but which nevertheless have an impact on the bacterial communities and our immune system: the eukaryotic microbes and viruses. This is particularly true of recent examinations of the microbiotas of the human colon, mouth, and vagina, but is also relevant to the microbiotas of other areas.

From recent metagenomic studies, fungi and bacteriophage play a significant role in the human health, but the DNA-based analyses of these components of the oral, vaginal, and colonic microbiota (the mycobiome and virome, respectively) have been relatively understudied. There are several challenges that have hampered progress in this area. Fungi are normally found as minor components of the healthy microbiota (overgrowth is usually associated with disease). Many fungi are difficult to cultivate or are uncultivable. It is also often difficult to lyse and extract genomic DNA from many fungi. To capture the mycobiome, recent metagenomic sequencing efforts have used the **internal transcribed spacer (ITS)** region of the rRNA genes as a basis for comparative sequencing analysis, analogous to the 16S rRNA sequencing analysis in bacteria.

Like the mycobiome, the virome (viruses and phage) has also been understudied. Again, there are a number of key challenges that need to be overcome for this field to move forward. Despite the fact that viruses are relatively abundant in numbers, they only make up a small portion of the total DNA or RNA in a sample. Virions are difficult to isolate, purify, and study, particularly in terms of finding suitable host cells for viral propagation. From those sequencing studies that have been undertaken, it appears that the bulk of the sequences are completely novel, with few, if any, homologs in the databases. This makes sequence annotation and comparative genomic analyses difficult to perform and, more significantly, it makes viral classification, evolution, and characterization within complex mixtures a daunting task. Nevertheless, many valiant efforts are currently underway.

In developing countries especially, there is another important eukaryotic component of the colonic microbiota that consists of protozoa and helminths (e.g., tapeworms). We tend to think of protozoa and helminths as pathogens, but this picture may not be entirely correct. Many people who carry these microbes are not sick. In fact, the eukaryotic component of the microbiota has been a fact of human life for millions of years. In areas of the world where such eukaryotic microbes are endemic, a majority of the population maintains them without any adverse effects. Only during the past century, and only in more developed parts of the world, has the eukaryotic component of the microbiota been virtually eliminated due to clean water, better hygiene, and a high-quality food supply.

There is some evidence that the abrupt (in evolutionary terms) loss of the eukaryotic component of the microbiota by people who live in developed countries may have contributed to a number of adverse effects, such as allergies and IBD. One current hypothesis is that helminths, and possibly protozoa as well, stimulate the arm of the immune system that consists of IgE, eosinophils, mast cells, and other cell types that are negatively associated with allergies and IBD. Support for this hypothesis arises from the fact that early stimulation of the gastrointestinal immune system by eukaryotic microbes allows this part of the immune response to develop normally. Conversely, failure to experience this type of stimulation may, in some people, predispose them to disease caused by overstimulation of the inflammatory response. The impact of the loss of these eukaryotic microbes on the bacterial microbiota has not yet been explored thoroughly, but efforts are now moving in this direction.

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Questions

1. How does a DNA-based analysis, such as the 16S rRNA gene analysis, differ from a cultivation-based analysis? In this chapter, the DNA-based approaches have been emphasized. What are some advantages of the cultivation-based approach?
2. More and more research groups are seeking to show that changes in the microbiota of a particular site are involved in diseases such as periodontal disease, inflammatory bowel disease, and premature birth. Critics object that showing an association is not the same as demonstrating cause and effect. In the case of the obesity study, scientists tried to do this by inoculating germfree mice with different variations of the microbiota. Clearly, this would not be possible in humans. How might you prove cause and effect in humans?
3. Infants in the first years of life are often more susceptible to certain bacterial infections than older children. How can you explain this? What function of the microbiota does this illustrate?

4. Members of the microbiota cause some quite serious diseases. How could a bacterium that normally lives in a beneficial or neutral association with its human host cause serious disease?

5. Metabolic interactions between members of the microbiota are attracting more attention because two microbes working together can increase the effectiveness of a reaction catalyzed by one of them. Consider an association between a polysaccharide-fermenting microbe and a methanogen in the colon. Consider also that the overall energy of a reaction depends on the ratio of substrate to end products for a bacterium like a polysaccharide fermenter. Can you explain why a polysaccharide fermenter and a methanogen might team up in the colon?

6. The assertion is made in this chapter that scientists now believe that transfer of DNA by conjugation in the colon is occurring across species and genus lines. Suppose you found the same type of antibiotic-resistance gene in members of two different genera. What criteria might you use to show that the gene was transferred horizontally? How might you suspect that the gene was transferred by conjugation?

7. Conventional wisdom asserts that there are no methanogens in the vaginal tract. If they were present they would probably be present at low levels. How would you use the 16S rRNA approach to find them? What modification of the approach used to find bacterial sequences would you have to make?

8. PCR combined with sequencing can provide a quick identification of bacteria. What are the limitations of this approach?

9. Resident microbiotas provide protection from colonization of some pathogenic bacteria in certain parts of the body. Describe regions of the body where normal microbiotas are protective and how they accomplish this protection.

For each of the following, choose the most correct answer:

10. The “normal” microbiota of humans is

    a. constantly changing.

    b. commensal.

    c. the same in every individual.

    d. parasitic.

11. Removing most of the microorganisms from the intestinal tract would

    a. inhibit infections.

    b. have no effect on health.

    c. lead to diarrhea and maybe other diseases.

    d. improve digestion.

12. Which of the following statements about normal microbiotas is not true?

    a. Our bodies begin to acquire a normal microbiota within a week of birth.

    b. Microbial communities, once established, remain constant throughout one’s lifetime.

    c. At the cellular level, our bodies are approximately 50% bacteria.

    d. Normal microbiotas can cause disease in humans.

13. *Clostridium difficile* is an example of

    a. how antibiotic use can cause a disease by disturbing the gut microbiota.

    b. how a shift in the bacterial microbiota of the gums can cause disease.

    c. how a shift in the eukaryotic microbiota of the vaginal tract can cause disease.

    d. a bacterium that is capable of surviving in the human stomach.

    e. a bacterium that is normally carried in the human nose.

14. *Helicobacter pylori* is

    a. a major component of the microbiota of the human small intestine.

    b. a cause of ulcers in the human stomach.

    c. responsible for most of the lactic acid production in the stomach.

    d. allows yeast to overgrow after antibiotic treatment.

    e. associated with gum disease and bad breath when a microbiota shift occurs.

15. The reservoir hypothesis refers to the

    a. effect of antibiotics on the composition of the oral microbiota.

    b. transfer of genes among bacteria in the human colon.

    c. shifts in bacterial populations that are associated with diet.

    d. differences in the prokaryotic-to-eukaryotic ratio.

    e. presence of Gram-positive bacteria in the vaginal tract.

16. A normal microbiota in the duodenum is
    a. similar to the microbiota in the stomach.
    b. similar to the microbiota in the colon.
    c. tolerant to acidic environments.
    d. both A and C.
    e. both B and C.
17. In the absence of the full complement of the normal microbiota, due to orally taken antibiotics, opportunistic microorganisms such as ________ can become established and cause disease.
    a. *Clostridium difficile*
    b. *Proteus mirabilis*
    c. *Staphylococcus aureus*
    d. all of the above
    e. none of the above

Solving Problems in Bacterial Pathogenesis

1. You are a researcher working for the U.S. Department of Agriculture. After two years of effort, you have isolated in pure culture a new, highly virulent bacterium from duck feces that is responsible for several major outbreaks of deaths in mammalian wildlife from contaminated pond water in the South. Based on 16S rRNA sequence comparison, you have determined that this new bacterium is distantly related to the Gram-negative bacterium *Vibrio cholerae*, and you have named the new strain *Vibrio birdsii*. You find that ducks are apparently unaffected by *V. birdsii*. You suspect that *V. birdsii* may be part of the normal microbiota of ducks. To test this hypothesis, you set up an experiment to examine the host response to *V. birdsii* in germfree ducks. The results are summarized in the graph shown in Figure 1. Provide a detailed explanation and interpretation for the results. Do the results support the hypothesis? Provide a rationale for your answer.

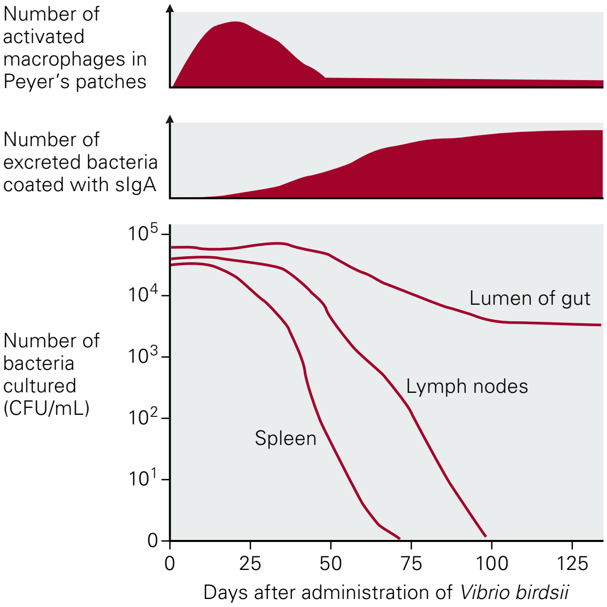

Figure 1. **Results.**

2. Dental plaque is a biofilm consisting of a complex community of over 700 different bacterial species. Epidemiological evidence suggests that a population shift toward certain Gram-negative anaerobes is responsible for initiation and progression of periodontal diseases. *Tannerella forsythia*, a Gram-negative, filamentous, nonmotile, anaerobic bacterium, is also considered one of the pathogens implicated in contributing to advanced forms of periodontal disease in humans and is strongly associated with cases of severe periodontitis. It is found co-aggregated in periodontal pockets with other putative periodontal pathogens, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Infection with *T. forsythia* induces alveolar bone resorption in a mouse infection model, in which the bacterium is inoculated under the gums of mice, followed by measuring for loss of dental bone. Considering that periodontal disease might be a community shift-type disease with multiple microbial participants, how might you use microbial community profiling methods to demonstrate the importance of the microbial community composition in contributing to the onset and maintenance of the diseased state? Set up the experiment first without using DNA sequencing approaches and then with using DNA sequencing approaches. Be sure to provide your rationale for method choice. From your results, how could you distinguish between a model of disease caused by a microbial community shift involving multiple microbes and one involving a single pathogen, such as *T. forsythia* or *P. gingivalis,* or a combination of both *T. forsythia* and *P. gingivalis*?

6

IN THIS CHAPTER

- **History and Relevance of Koch's Postulates**
  - Early Germ Theory
  - Koch's Postulates: A Set of Criteria Used To Establish a Microbe-Disease Connection
- **Challenges to Satisfying Koch's Postulates**
  - Easier Said than Done. . .
  - The First Postulate: Association of the Microbe with Lesions of the Disease
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  - The Fourth Postulate: Reisolating the Bacterium from the Intentionally Infected Animal
- **Modern Alternatives To Satisfy Koch's Postulates**
  - Detecting the Presence of the Pathogen Only in Diseased Tissues
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  - Views of the Human-Microbe Interaction
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CHAPTER 6

Microbes and Disease

ESTABLISHING A CONNECTION

"*Felix, qui potuit rerum cognoscere causas*" ["Fortunate is he, who is able to know the causes of things."]—verse 490, Book 2 of *Georgics*, Virgil (circa 29 BCE). Modern microbiologists seeking to cure previously intractable diseases have embraced the spirit of this quote by the famous Latin poet. Indeed, one of the most exciting areas of infectious disease research was kicked off by research into the cause of gastric ulcers and the discovery of their bacterial origins. For decades, physicians believed that gastric ulcers were a chronic condition caused by genetic predisposition and excess stomach acid production, which thus led to the conclusion that ulcers could not be cured and that it was only possible to treat the symptoms through bland diets and daily doses of antacids. The finding that most gastric ulcers are caused by a particular bacterium led at long last to a cure for ulcers—fortunate indeed for all chronic ulcer sufferers who now could go back to eating their favorite spicy foods. Now scientists are enthusiastically exploring the possibility that bacteria might be the cause of other chronic conditions, such as inflammatory bowel disease, cardiovascular disease, atherosclerosis, and certain cancers, which, if so, might also be curable. The problem is demonstrating that the bacterium is indeed the direct cause of the disease and not simply associated with the condition. Scientists have been grappling with this problem for over a century, and even today with a strong set of guidelines such as Koch's postulates in hand, finding a clear-cut connection between microbe and disease is challenging. Today, researchers are applying modern molecular technologies to tackle the microbe-disease causation problem.

History and Relevance of Koch's Postulates

Early Germ Theory

The first sightings of microbes through the microscopes invented by Antonie van Leeuwenhoek (1632–1723) occurred in the 1600s, but it took two centuries for the connection between observed microbes and infectious disease to be solidified. This lag is not surprising given the relative sizes of humans and microbes. How, asked early skeptics, could something that was too small to be seen by the unaided eye possibly sicken and kill human beings? Today, we take the connection between microbes and infectious diseases for granted, but this connection was not accepted easily at the time.

When the **germ theory of infectious disease** appeared in the middle of the 19th century, it challenged and eventually replaced the **miasma theory**, which had been the predominant explanation for the spread of infectious diseases since the Middle Ages. The miasma theory was a belief that infectious diseases were caused and transmitted by foul vaporous emanations that were associated with decaying matter and bad sanitary conditions. We still have a remnant of this notion in the name "malaria," which appeared in 1740 and literally means "bad air" in Italian. Champions of the miasma theory, notably Max J. von Pettenkofer (1818–1901), realized that cleaning up sewage and water significantly reduced diseases such as cholera and typhoid. Improved cleanliness removed bad-smelling fens and reduced disease rates, but this only further reinforced the miasma theory to its proponents. Although carried out for the wrong scientific reasons, the miasma theory did lead to some notable successes in public health reform and improved hygiene.

In the 21st century, it is somewhat hard to imagine that the germ theory of disease was so controversial when it was first proposed. Agostino Bassi (1773–1856), Friedrich Gustav Jacob Henle (1809–1885), Louis Pasteur (1822–1895), and other early advocates of germ theory realized that the connection between a microbe and a particular set of symptoms needed to be established on a sound scientific foundation. After all, Pasteur was the scientist whose earlier work on fermentation processes led to improved processing methods now known as **pasteurization**, which involves brief heating to kill most bacteria and fungi and thereby prevents spoilage of beverages, such as beer, wine, and milk. His findings led to further studies that disproved the other widely held notion of **spontaneous generation** by his elegantly simple "swan-neck" flask experiment (Figure 6-1).

The germ theory of disease was further bolstered by the work of Ignaz Semmelweis (1818–1865) that demonstrated the effectiveness of handwashing in preventing childbirth fever (see Box 2-2). The germ theory also provided a scientific explanation for the horrendous infection rates that occurred during surgery in the 19th century, where handwashing was not practiced routinely due to the prevailing belief at the time about the airborne transmission of disease. Joseph Lister (1827–1912) and others seized on the germ theory to develop antiseptics and sterilization methods for surgery that greatly improved survival rates. With these early studies in hand, a number of scientists were becoming more receptive to the notion of a microbial origin of infectious disease, which set the stage for establishing the principles governing how to make the microbe-disease connection.

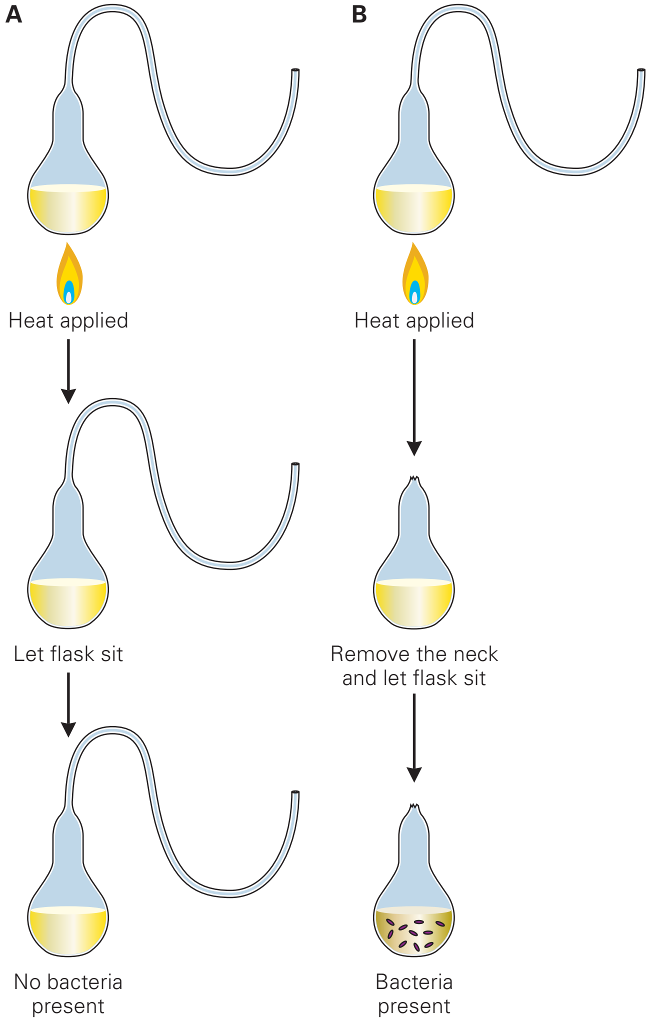


Figure 6-1. Louis Pasteur's "swan-neck" flask experiment. In 1862, Louis Pasteur conducted the first experiments demonstrating that the fermentation and growth of microorganisms in nutrient broth did not occur by spontaneous generation, as was commonly believed at the time. In this experiment, Pasteur prepared two swan-neck flasks, adding nutrient broth and then boiling it to sterilize the broth. One of the flasks he left intact, but he broke off the top of the S-shaped neck of the other flask, exposing the broth to air from above. With time, the flask with the top exposed to air from top became cloudy from the growth of microorganisms in the broth, whereas the intact swan-neck flask remained clear and microbe free, even though it was still exposed to air.

Koch's Postulates: A Set of Criteria Used To Establish a Microbe-Disease Connection

The German microbiologist Robert Koch (1843–1910) sought to define a set of rules for establishing a connection between a particular microbe and a particular disease. Early versions of his proposed guidelines, published first in 1882 and revised in 1884, consisted of three criteria that needed to be satisfied to link a microbe with a disease. In his final version, published in 1890, Koch refined his proposed set of rules by adding a fourth criterion. These four criteria for establishing cause and effect came to be known as **Koch's postulates** (Table 6-1).

Koch formulated his postulates based on elegant and detailed studies that he conducted over many years, beginning with his earlier work on anthrax and the life cycle of its causative bacterium, *Bacillus anthracis*. Although *B. anthracis* is now widely recognized as a potential biological weapon (see chapter 20), this bacterium is found in the environment, where it infects and kills livestock and wild herbivores (Figure 6-2). In addition to the concept of germ theory, several other key developments played a role in setting the stage for Koch to advance his postulates. These were his microscopic studies of microorganisms, his advancements on improved techniques for staining bacteria in tissues, and his development of new pure-culture isolation methods. Importantly, Koch's use of these experimental methods in his studies with animal models of disease culminated in his demonstration of the bacterial origin of anthrax and later tuberculosis, which helped him refine his postulates as well as provide evidence for their validity.

Koch's first postulate states that the microbe must be associated with all lesions of the disease. That is, the microbe should be present in all cases where symptoms of disease are evident and found in diseased tissue but not in healthy tissue.

Table 6-1. Koch's postulates: then and now

Original Postulates Proposed by Koch:

1. The microbe must be associated with symptoms of the disease and must be present at the site of infection.
2. The microbe must be isolated from the lesions of disease and grown as a pure culture.
3. A pure culture of the microbe, when inoculated into a susceptible host, must reproduce the disease in the experimental host.
4. The microbe must be reisolated in pure culture from the experimentally infected host.

Alternative Methods that Can Be Used to Satisfy Koch's Postulates:

Detection

Premise: Modern molecular biological techniques can be used to demonstrate the presence of the microbe in diseased lesions, but not in healthy tissue.

- PCR can be used to detect and identify the microbe in diseased tissue.
- Immunohistochemistry or immunofluorescence microscopy can be used to detect and identify the microbe in diseased tissue.

Reduced Exposure

Premise: Preventing exposure to or reducing the amount of a pathogen should protect against infection and spread of disease.

- Hygiene, disinfection, and health practices can be used to prevent exposure to the microbe and thereby prevent disease.

Elimination of Microbe

Premise: Elimination of the disease-causing microbe from the infected host should prevent infection and spread of disease.

- Antibiotic therapy can be used to eliminate the microbe and thereby cure the disease.
- Vaccination can be used to prevent infection by the microbe and thereby prevent disease.

Comparative Infectious Disease Causation

Premise: If naturally occurring chronic infection by a disease-causing microbe results in manifestation of similar symptoms or disease sequelae in multiple mammalian genera, then cause and effect has been established for that microbe.

- Comparative infectious disease causation associated with chronic infection of a particular stealth microbe in multiple mammalian genera can be used to link that microbe with a particular disease.

Bradford Hill's Criteria for Causation

Proposed in 1965 by the English epidemiologist Sir Austin Bradford Hill (1897–1991), these criteria are often used for defining causal relationships in epidemiological studies. These criteria have served as guidelines for epidemiological aspects of evidence-based medicine, which is the application of scientific inquiry to inform clinical decisions.

- Strength of the association: the larger the association is, the more likely it is causal.
- Consistency: the more reproducible the association is, the more likely it is causal.
- Specificity: the more specific the association between a factor and an effect is, the more likely it is causal.
- Temporality: the effect can be observed only after the cause has occurred.
- Biological gradient: the greater the exposure is, the greater the effect should be and the more likely it is causal.
- Plausibility: a plausible explanation between cause and effect should be found, and if it is, then the more likely it is causal.
- Coherence: findings from epidemiological studies must be consistent with experimental data from laboratory studies.
- Experimental evidence: there should be a preponderance of experimental data supporting the causal relationship.
- Analogy: other similar factors should cause similar effects.

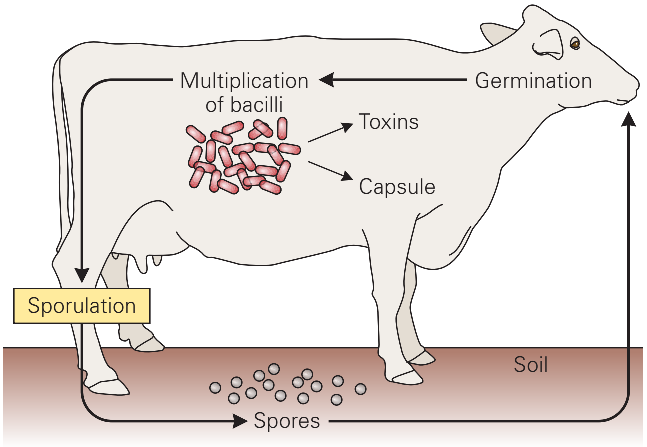


Figure 6-2. Life cycle of *Bacillus anthracis* and anthrax. Anthrax disease is caused by Gram-positive, spore-forming *Bacillus anthracis*, which can live in the soil for many years as heat-resistant endospores. While humans and other animals are susceptible to infection, domestic livestock and wild herbivores are especially vulnerable because they can ingest the spores along with the grasses they feed on. The ingested spores germinate within the herbivores to convert into the vegetative bacilli that replicate and express virulence factors (anthrax toxins and capsule), which then kill the host. Bacilli released by the dying or dead animal will then sporulate upon being exposed again to air and environmental conditions outside the body. Adapted from Mock M, Fouet A. 2001. *Annu Rev Microbiol* **55:**647–671, with permission.

The second postulate directs that the microbe must be able to be isolated from the lesions of the disease and grown as a pure culture of a single organism. The third postulate states that a pure culture of the isolated microbe should reproduce the symptoms of the disease if it is inoculated into a susceptible host, either human or experimental animal. The fourth postulate states that the same microbe must be able to be reisolated in pure culture from the experimentally infected host that was used to satisfy the third postulate.

Challenges to Satisfying Koch's Postulates

Easier Said than Done. . .

Since their first publication, Koch's postulates have proven invaluable for demonstrating that infectious diseases are caused by microbes. In addition, the experimental methods developed by Koch and his coworkers to satisfy the postulates have had a lasting influence on the practice of microbiology, including the isolation and cultivation of bacterial colonies on agar medium contained in a petri dish, which was named after one of Koch's assistants, Julius Richard Petri (1852–1921). However, while the postulates sound very reasonable and straightforward and have shown a high success rate for identifying and evaluating a large number of bacterial pathogens, it quickly became clear that they can be difficult to satisfy in some cases and may not apply universally to all infectious diseases.

First, Koch's postulates assume that the disease symptoms depend entirely on the bacterium, yet host susceptibility varies in human and animal populations. It is now well established that host genetics, environment, and other factors, such as age and proficiency of the immune system, play a major role in the extent of pathology observed. Some individuals can be colonized with a potential disease-causing bacterium yet not develop any overt symptoms of the disease, whereas others are severely affected. Not all individuals respond equally to an infectious microbe, so the disease may not take the same course of progression in all individuals. In addition, prior infection with one microbe may influence the response of the host to subsequent infection with another microbe.

Second, the postulates assume that a pathogenic bacterium can be isolated and cultured readily in pure form. Unfortunately, not all bacteria can be cultured (or at least we have not yet determined how to culture them) under standard laboratory conditions. But even when they can, they may accumulate genetic changes upon being cultured that reduce some of the traits that allow them to cause disease. We now know that many bacteria can undergo gene shuffling or regulate their virulence genes such that the virulence properties may change with time or with exposure to different environments. Indeed, many pathogens change the expression of their virulence traits in response to whether they are inside or outside the host and where they are in the host.

Third, Koch's postulates assume that all members of a bacterial species are equally virulent and that a single species causes each disease. This is clearly not the case for all bacterial pathogens. There can be dramatic and distinct differences in disease-causing ability, even between closely related bacterial strains. As we shall see in later chapters, most bacteria can readily exchange genetic material and thereby alter their virulence properties.

Finally, Koch's postulates require reinoculation into a susceptible host to reproduce the disease symptoms. For human disease, this requires either brave volunteers in highly structured studies or, more likely, a good animal model. However, a suitable animal model may not be available, and for many diseases nonhuman animals are indeed only approximate model systems for human disease.

As a consequence of these apparently intractable issues, there remain a number of infectious microbes for which all of Koch's postulates have not yet been satisfied completely, despite general agreement in the field that a particular microbe is or is highly likely to be responsible for the given disease. To illustrate why these issues might make it difficult to satisfy Koch's postulates, it helps to consider some examples of attempts to prove that a particular bacterium causes a particular disease. We will explore a selected number of these cases in the following sections for each of the postulates.

The First Postulate: Association of the Microbe with Lesions of the Disease

It was relatively straightforward for Koch to demonstrate the presence of the large *B. anthracis* bacilli in anthrax lesions and to later demonstrate that the heat-resistant (and hence pure) anthrax spores also caused disease. But the small, slow-growing bacterium that causes tuberculosis required the development of more sophisticated methods before Koch was able to satisfy his first postulate for this microbe.

Microbiologists are now well aware that *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is not only difficult to culture but also difficult to stain using crystal violet, the dye used for Gram staining bacteria. At the time (early 1880s), Koch was convinced that the causative agent of tuberculosis was a bacterium because he could induce similar disease pathology in guinea pigs by inoculating tuberculous material from various clinical sources in the animals and show that this resulted in disease. The problem was that he could not actually see the bacteria in diseased tissues by using Gram staining. This spurred him to develop an early version of the **acid-fast stain**, where alkaline methylene blue dye was used to penetrate the waxy cell wall of the bacterium, followed by destaining with dilute hydrochloric acid. Shortly after, Franz Ziehl (1857–1926) and Friedrich Neelsen (1854–1898) further improved this acid-fast staining procedure by adding the red dye carbol fuchsin, which could better penetrate the mycobacterial cell wall. After staining using this new procedure, acid-fast bacilli are stained bright red, while non–acid-fast bacteria are blue (Figure 6-3). Thus, Koch's success in demonstrating that a bacterium was responsible for tuberculosis depended not only on his development of special bacterial cultivation methods but also on the discovery of a method for staining and detecting the mycobacteria in tissue samples.

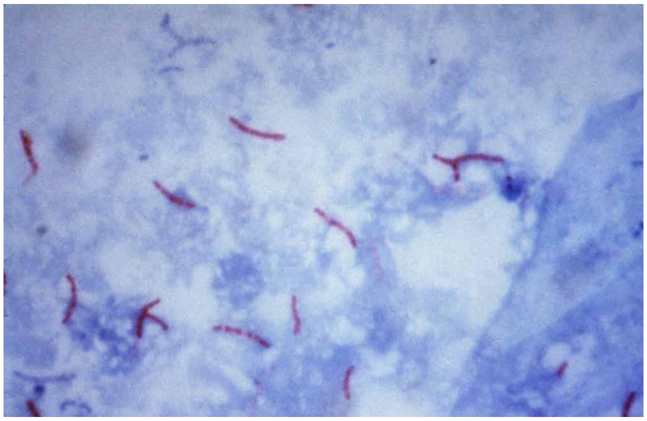


Figure 6-3. Detection of *Mycobacterium tuberculosis* by Ziehl–Neelsen acid-fast staining. Shown is a micrograph of acid-fast–stained *M. tuberculosis* (red). Image courtesy of CDC/Dr. George P. Kubica (CDC-PHIL ID#5789).

Strictly satisfying the first postulate has not always been possible, even when a causal relationship is eventually established between a microbe and a disease. For example, it took over 10 years for the scientific community to accept the idea that gastric ulcers could be caused by *Helicobacter pylori*. The reason for the skepticism was the fact that not all cases of colonization with *H. pylori* are associated with symptoms of disease. Over half of the people in developed countries and nearly all of the people in developing countries carry *H. pylori* in their stomachs, but only a small number of colonized people develop ulcers. Likewise, colonization with *H. pylori* could not be demonstrated for all cases of gastric ulcers. As an aside, it is worth pointing out that this same pattern is seen for many other bacterial diseases, where the bacterium colonizes many people, but only those with some predisposing condition develop overt disease.

Koch himself was one of the first to worry about his first postulate, as it became obvious from his studies and those of others that people could be colonized with a pathogenic bacterium without having any symptoms (the **carrier state**). We now know that the genetic makeup as well as the current and prior health of the host plays a critical role in the duration and severity of disease and in the recovery of an individual from an infection. We also know that disease outcome is not always predictable and that disease progression is dynamic and depends on many microbial and host factors. In some cases where a clear-cut causal relationship has yet to be established, infection with the microbe may represent a risk factor instead of a cause (Table 6-1).

An example of this situation is the apparent link between the bacterium *Chlamydophila pneumoniae* (formerly called *Chlamydia pneumoniae*) and the heart diseases atherosclerosis and coronary artery disease. Scientists first began to suspect that bacteria might cause atherosclerosis when they isolated *C. pneumoniae* from atherosclerotic plaques but seldom isolated the bacteria from healthy blood vessel tissue. Occasionally, however, *C. pneumoniae* was isolated from healthy tissue. Finding *C. pneumoniae,* a common cause of mild respiratory infections, in healthy tissue is not surprising if the characteristics of the bacteria are considered. *C. pneumoniae* invades and lives inside human cells, thus protecting itself from the body's defenses. So, if bacteria from the throat of a person with a respiratory tract infection leaked into the bloodstream, they might well infect blood vessel cells transiently and thus be found in tissue that looks healthy. The fact that *C. pneumoniae* was occasionally isolated from healthy tissue may be rationalized in this way, but it blurs the clear line implicit in Koch's first postulate. Even worse, although *C. pneumoniae* could be isolated from atherosclerotic plaque tissue, it was not isolated from all such tissues.

Thus, *C. pneumoniae* was often associated with lesions of the disease, but not all of the time.

The Second Postulate: Isolating the Bacterium in Pure Culture

Koch was well aware that the presence of a microbe did not, in and of itself, prove that that particular organism was the actual cause of the disease being manifested. He knew he first had to isolate the bacterium in pure culture from the diseased tissue before he could demonstrate that it was the primary cause of the disease. However, like the first postulate, the second postulate is also not as easy to satisfy as it sounds. As was pointed out in the first chapter, bacteria are extraordinarily diverse with respect to their metabolic traits and growth requirements. Bacteriologists who work with disease-causing bacteria frequently grow these bacteria by streaking them onto a complex agar medium and incubating the agar plates with ordinary air as the atmosphere. But, while this medium works for many bacteria, some bacteria are more difficult to cultivate than others under these conditions.

Koch also ran into this problem when he began his work on tuberculosis. He spent over six years developing his culture media formulations and plating techniques, which worked well for *B. anthracis* and other bacteria that cause wound infections. However, these methods did not work for *M. tuberculosis*, which did not grow at room temperature and only very slowly at body temperature. Because the nutrient gelatin he used for the plates melted at body temperature, he had to develop another method that instead used coagulated blood serum, upon which after about a week the bacteria formed tiny colonies that could be seen under a low-power microscope.

Microbiologists have developed a wide variety of formulations for liquid media or agar plates over the years, such that we now are able to cultivate a very large number of diverse bacteria. However, although there are some media that can support the growth of a wide variety of bacteria, there is no universal medium on which all bacteria can be cultivated. Indeed, all of these approaches have failed to cultivate *C. pneumoniae*. Because *C. pneumoniae* only grows inside human cells, it must be cultivated using tissue culture cells and not agar medium. Moreover, an atmosphere with an elevated CO_2 content is required for optimal growth. The fact that *C. pneumoniae* is more difficult to cultivate than many other disease-causing bacteria raises the question of whether the failure to cultivate *C. pneumoniae* from all atherosclerotic lesions could actually be due to the failure of the bacteria that might be present in a lesion to grow in all cases. Likewise, although *H. pylori* can grow on a select agar medium, it also requires a special atmosphere, and it took a lot of effort to find conditions that would support its growth.

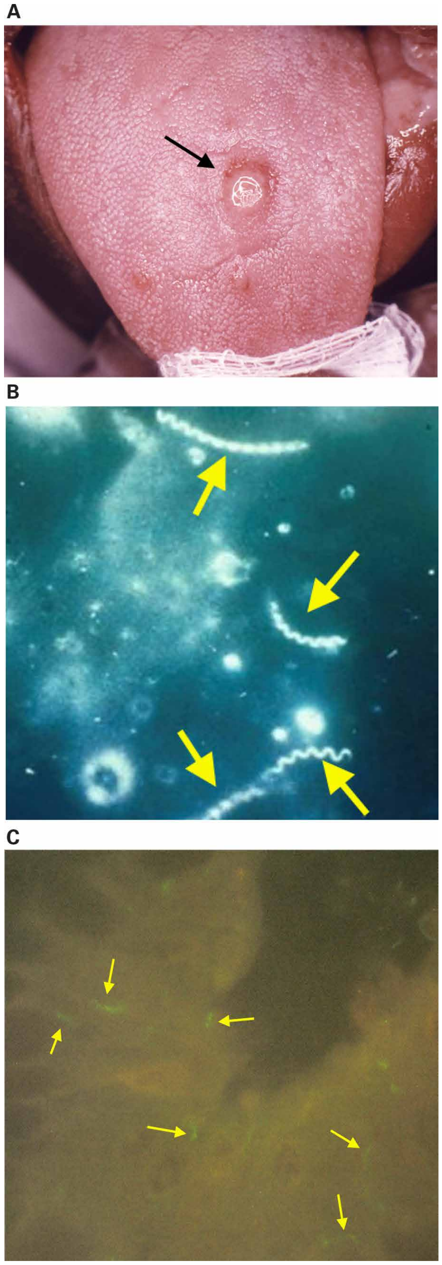


Figure 6-4. Visualization of *Treponema pallidum* in syphilitic lesions. The spirochete *Treponema pallidum* can be seen in samples taken from syphilitic lesions. **(A)** Primary syphilitic lesion (arrow) on the surface of a tongue. **(B)** Micrograph of the *T. pallidum* spirochetes (arrows) taken under dark-field microscope. **(C)** Fluorescence micrograph of human gastric tissue from a patient with syphilis, where the spirochetes are detected using green fluorescent dye-labeled monoclonal antibodies generated against antigens from *T. pallidum* (arrows). Panels A and B courtesy of Division of STD Prevention, National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention, Centers for Disease Control and Prevention. Panel C from Ito F, Hunter EF, George RW, Pope V, Larsen SA. 1992. *J Clin Microbiol.* **30:**831–838, with permission.

An extreme example of the difficulty that can arise in attempting to satisfy Koch's second postulate is provided by *Treponema pallidum*, the bacterium that is generally accepted as the causative agent of the **sexually transmitted disease (STD) syphilis**. The *T. pallidum* bacterium has a distinctive helical or corkscrew shape (hence the name "spirochete") and can be seen under a microscope in lesions associated with the early stages of syphilis (Figure 6-4). Yet *T. pallidum* has never been cultivated as a pure culture in laboratory medium, despite exhaustive attempts. The closest thing we currently have to a laboratory medium for *T. pallidum* is the rabbit testicle, which is used as a growth chamber by scientists who work on *T. pallidum*.

The Third Postulate: Showing that the Isolated Bacterium Causes Disease Experimentally in Humans or Animals

The third postulate demands that the bacterium must produce the disease when inoculated experimentally into a human or an animal. Around the time that Koch was showing that *B. anthracis* caused anthrax in animals, his colleague Ferdinand Cohn (1828–1898) was characterizing another bacterium that he had isolated from hay and that did not appear to cause disease. This bacterium, which he called *Bacillus subtilis,* looked like *B. anthracis* under a microscope, could form spores just like *B. anthracis,* and possessed many similar morphological and biochemical properties as *B. anthracis* when grown in culture. Both researchers realized that an organism could not be considered the cause of a disease just because it looked like the disease-causing bacterium under a microscope or exhibited some similar biochemical or physiological properties. They agreed that proof could only come after the critical demonstration that the isolated bacterium, when inoculated into a susceptible host, caused the disease. Koch subsequently showed that purified spores of *B. anthracis*, but not spores of *B. subtilis*, caused anthrax in experimental animals. Koch published his findings in Cohn's journal as paper number 5 in a series of articles, following Cohn's own paper number 4 on the characterization of *B. subtilis*.

Later, Koch was also able to satisfy this critical third postulate for *M. tuberculosis* using a guinea pig infection model. Even though guinea pigs do not naturally get infected with or succumb to tuberculosis, they do exhibit similar disease pathology and die from tuberculosis when inoculated experimentally with pure cultures of *M. tuberculosis* isolated from diseased human tissues.

Of all of Koch's postulates, this third postulate has been the most difficult to satisfy for many pathogens. Ironically, one of the first critics of this postulate was Koch himself. Less than two years after Koch first proposed his postulates, he ran afoul of them when he tried to tackle cholera (Box 6-1). The ill-fated von Pettenkofer is also reported to have tried to infect himself by drinking a culture of *V. cholerae* that he

Box 6-1.

Koch Backs Off

People sometimes grumble about criticisms of Koch's postulates. After all, they say, Koch's postulates are all we have. What they may not realize is that the first person to give the old heave-ho to Koch's postulates, or at least the third one, was Koch himself. The ink was barely dry on the paper before he was having second thoughts. Koch was passionately interested in the disease cholera and was convinced he had found the causative agent, a curved bacillus ("comma bacillus" to Koch, now known as *Vibrio cholerae*). Through numerous dissections of cholera victims, he had consistently observed and isolated the comma bacilli from diseased intestines. He had also conducted epidemiological and research studies of cholera outbreaks in Egypt and India, which only reinforced his convictions. It turned out that Koch was right, but in 1884 his own postulates were being used against him because there was no animal model for cholera.

Here is what Koch had to say to his critics at the time:

". . . no one ever observes animals with cholera. Therefore, I believe that all the animals available for experimentation and those that often come in contact with people are totally immune. True cholera processes cannot be artificially created in them. Therefore, we must dispense with this part of the proof. This certainly does not mean that there is no proof that comma bacilli are pathogenic. I have already mentioned that even without animal experiments, I can imagine nothing other than that the comma bacilli cause cholera. If a cholera process is finally produced in animals, I will be no more convinced than I am now."

Source:

Haffajee AD, Socransky SS. 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* **5:**78–111. In this paper they are quoting from a translation of Koch's essays [**Carter KC**. 1987. *Essays of Robert Koch*, p. xvii–xix, 161. Greenwood Press, Westport, CT] in defense of their proposed alternatives to Koch's postulates.

obtained from Koch. When this uncontrolled and dangerous self-experiment failed, it was interpreted by von Pettenkofer and other miasma theory adherents as evidence against the germ theory.

It is impressive that Koch was far more flexible in his approach to proving cause and effect in infectious disease than some modern-day scientists. In recent years, the third postulate has been a stumbling block for scientists working on *H. pylori* and *C. pneumoniae.* Acceptance of the proposal that most ulcers are caused by bacteria was held up for nearly a decade because critics of the idea insisted that Koch's third postulate had to be met. There are now good animal models for ulcers, but in the early days of *H. pylori* research these models were not available. Indeed, the lack of an animal model led one frustrated scientist to again use himself as the guinea pig in an attempt to satisfy Koch's third postulate (Box 6-2).

One of the early animal models for gastric ulcers was a ferret inoculated with *Helicobacter mustelae*. While *H. mustelae* is a close relative of *H. pylori,* critics argued that it was not *H. pylori* and that disease caused by it in an animal model could not be considered the equivalent of the human disease. Similarly, many researchers today use infection with *Salmonella* Typhimurium (now more properly called *S. enterica* serovar Typhimurium) in mice to mimic systemic disease caused by *Salmonella* Typhi (*S. enterica* serovar Typhi) in humans, because the human-specific *S.* Typhi does not cause typhoid-like disease in mice. But *S.* Typhimurium is a well-known cause of intestinal food poisoning in humans and does not usually cause systemic disease in humans. Is it acceptable to use a different bacterial species as well as a different animal species to satisfy Koch's third postulate? Scientists still differ in their answers to this question.

Scientists working on the proposed connection between *C. pneumoniae* and heart disease have used a breed of rabbit that is prone to develop atherosclerosis if fed a high-fat diet to show that infection with *C. pneumoniae* increases the

Box 6-2.
Koch's Postulates Are Not Just Words on Paper

How much do you care about proving a theory you believe in but cannot get others to accept? Barry Marshall, a young Australian internist working with J. Robin Warren, the discoverer of *H. pylori*, did not exactly put his life on the line to do so, but he placed himself at risk for a very unpleasant condition. Marshall was an early proponent of the hypothesis that many gastric and duodenal ulcers are caused by *H. pylori.* The idea that bacteria and not stress are the cause of ulcers flew in the face of well-established medical dogma (see Box 6-3). Given this, it is not surprising that Marshall and others who advanced this outrageous notion were not at first taken seriously by others working in the field. They were on firm ground in the beginning because all that had been proven was that *H. pylori* was associated with ulcer lesions and could be isolated in pure culture from these lesions (Koch's postulates 1 and 2).

Critics of Marshall's theory insisted that Koch's third postulate must also be satisfied (i.e., that the bacteria isolated from ulcers could cause ulcers in animals or humans). At the time, Marshall had been trying unsuccessfully to get the bacteria to infect various laboratory animals. Frustrated by this, he turned to the obvious alternative: human subjects. But who would volunteer to be inoculated with a bacterium that might cause ulcers? Keep in mind that this was before a successful antibiotic regimen had been developed. And even if volunteers could be found, the institutional committee overseeing the use of human subjects for research would deny approval for the experiment. Marshall thus turned to his most faithful and reliable supporter: himself. He first had his stomach checked by endoscopic examination to make sure that the stomach mucosa was healthy. Then, he drank a turbid culture of *H. pylori*, which had been recently isolated from the lesions of a patient with peptic ulcers. Within a few hours, his stomach began to growl. A week later, he became nauseated and vomited. During this period, he felt unusually hungry and tired. Near the end of the second week, he underwent a second endoscopic examination of his stomach and a biopsy was taken. A portion of Marshall's stomach had an inflamed appearance, and *H. pylori* were found in the mucin layer over the lesion. The bacteria Marshall had ingested were clearly capable of causing disease. Fortunately for Marshall, the infection healed spontaneously, but Marshall's point was proven.

According to an article in *The New Yorker* that describes Marshall's experiment and its aftermath in detail, the initial event of taking the bacterial dose, a potentially momentous occasion in the history of gastroenterology, was a disappointingly undramatic affair. A fellow worker in the lab, who knew what Marshall was about to do, said to him, "You're crazy." Marshall replied with "Here goes," and then drank the culture, remarking that it tasted like swamp water. Apparently, Marshall had spent too much time thinking about the experiment he planned to do to bother providing himself with a suitably memorable statement to commemorate the occasion. Because of their epochal discoveries showing *H. pylori* as a causative agent of ulcers, Marshall and Warren were awarded the Nobel Prize in Physiology or Medicine in 2005. For a full account, see **Monmaney T**. 20 September 1993. Marshall's hunch. *The New Yorker*, p. 64–72.

development of atherosclerotic plaques. However, this demonstration has not convinced many critics, who argue with some justification that the rabbit model is not a good replica of the disease in humans. Objections such as these raise the important issue of just how closely an animal model must mimic the disease in humans for it to serve as an acceptable infection model.

The Fourth Postulate: Reisolating the Bacterium from the Intentionally Infected Animal

Anyone who manages to satisfy the first three postulates will probably be able to satisfy the fourth one with little difficulty. Nevertheless, the fourth postulate is important. In satisfying the fourth postulate, a scientist shows that the human or animal used to satisfy the third postulate was actually infected and that the lesions of the experimentally inoculated human or animal, like those occurring in the natural disease, contained the microbe. For example, in the case of the rabbit model used to study whether *C. pneumoniae* would cause atherosclerotic plaques to form, one might object that the reason the plaques formed in the rabbit arteries was not because the arteries were infected by the bacteria, but rather that the bacteria caused a mild respiratory illness, which stressed the rabbits enough to indirectly increase the rate of plaque formation. Finding the bacteria in the experimentally induced plaques helps bolster the contention that their presence caused the increase in plaque formation.

Modern Alternatives To Satisfy Koch's Postulates

Do the above examples of problems that can be encountered when trying to satisfy Koch's postulates imply that Koch's postulates are useless?—Not at all!

For many diseases, especially those caused by bacteria that produce obvious virulence factors such as toxins, Koch's postulates can be rigorously met. In other cases, Koch's postulates are very useful for guiding the design of experiments to establish a connection as well as subsequent discussions about the interpretation of the experimental results. What the above examples show, and what Koch also realized at the time, was that these postulates should not be treated as monolithic requirements. To waive any one of Koch's postulates, scientists should certainly be expected to convincingly explain why failure to satisfy that postulate does not invalidate their claim for a cause-and-effect relationship. Not surprisingly, since Koch's time, scientists have developed a number of modern molecular approaches as well as additional guidelines with criteria that can be used to help provide evidence of cause and effect, thereby serving as alternative ways to satisfy Koch's postulates (see Table 6-1).

Detecting the Presence of the Pathogen Only in Diseased Tissues

A modern alternative to detecting the presence of pathogenic bacteria in diseased but not healthy tissues is to use molecular biology approaches, such as the polymerase chain reaction (PCR)-based amplification methods described in chapter 5. For this application, specific DNA primers directed against a specific pathogen amplify the pathogen's DNA, using PCR to detect the presence of the pathogen in the tissue (Figure 6-5). Notable advantages of this method are that it is highly sensitive for detecting minute quantities of the pathogen in a sample; it is very specific for a particular pathogen; and multiple samples and multiple pathogen species can be assayed simultaneously (**multiplexed**) using selective sets of primers. People who study *C. pneumoniae* often use multiplexed PCR analysis because it can distinguish among different *Chlamydophila* species and it is much faster and easier than selective cultivation of the bacteria, which is difficult and time-consuming.

Another modern approach used to help satisfy Koch's first postulate is to use **immunohistochemistry** to detect the bacteria in tissue samples (Figure 6-6). This method relies on the use of specific antibodies raised against the bacteria that are then conjugated to a reporter enzyme for use in a colorimetric assay to stain tissue samples for the presence of the pathogen. Alternatively, the antibodies could be conjugated to a fluorescent dye that could be used for visualization by **immunofluorescence microscopy**. Again, in each of these cases the bacteria would be detected or visualized in diseased tissue but not in healthy tissue.

Additional methods for detecting the presence of the pathogen in tissue or blood are variations of the **enzyme-linked immunosorbent assay (ELISA)**. There are three main variants of ELISA that are commonly used (Figure 6-7): (1) **Direct ELISA**—Antigens released from the microbe-containing sample are attached via **adsorption** to a surface, such as a microtiter plate well. Pathogen-specific antibodies are conjugated to a reporter enzyme and then applied to the antigen-coated surface. After incubation, the plate well is washed to remove any unattached antibodies, a solution containing the reporter enzyme's colorimetric substrate is added, and the resulting color signal is measured. (2) **Indirect ELISA**—Similar to the direct assay, the well surface is first coated with antigens from the pathogen, and then pathogen-specific primary antibodies are applied to the well. Unbound primary antibodies are washed away, and a secondary antibody that recognizes the primary antibody and is conjugated to a reporter enzyme is applied. The colorimetric assay is then conducted as before. (3) **Capture** or **Sandwich ELISA**—The microtiter plate surface is first coated with a capture antibody that specifically recognizes an antigen from the pathogen. The sample containing the pathogen is applied, and the surface-attached antibody captures the pathogen from the sample.

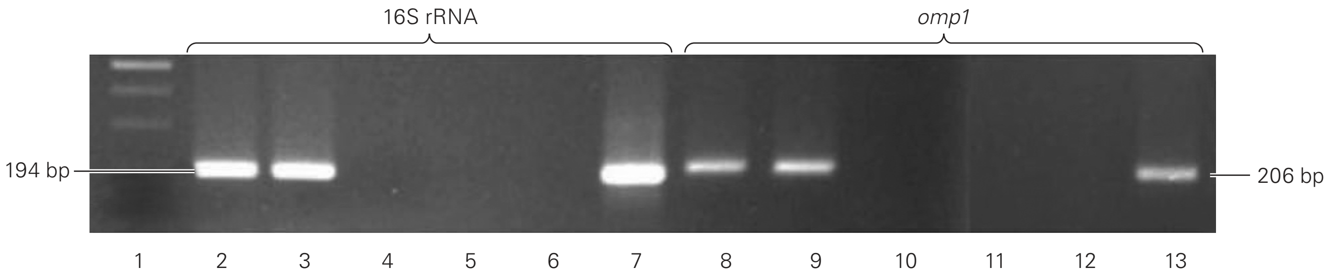


Figure 6-5. Detection of pathogen DNA in infected tissues by PCR-based amplification of pathogen-specific genes. A pathogen can be indirectly detected in infected tissues by PCR amplification of the pathogen's DNA using primers designed specifically for genes encoded by the particular pathogen. In this case, DNA extracted from human plasma was used as a template for PCR amplification using two different *C. pneumoniae*-specific primer sets, one pair designed for the 16S rRNA gene that resulted in a 194-bp PCR product and another pair designed for the gene *omp1*, which encodes the surface protein OmpA that resulted in a 206-bp PCR product. Shown is an image of the resulting ethidium bromide-stained agarose gel of the resulting PCR reactions. Lane 1: molecular size standards; Lanes 2 and 8: patient M; Lanes 3 and 9: patient P; Lanes 4 and 10: patient S; Lanes 5 and 11: extraction control; Lanes 6 and 12: negative controls; Lanes 7 and 13: positive controls. Adapted from Petyaev IM, Zigangirova NA, Petyaev AM, Pashko UP, Didenko LV, Morgunova EU, Bashmakov YK. 2010. *Int J Med Sci* **7**:181–190, with permission.

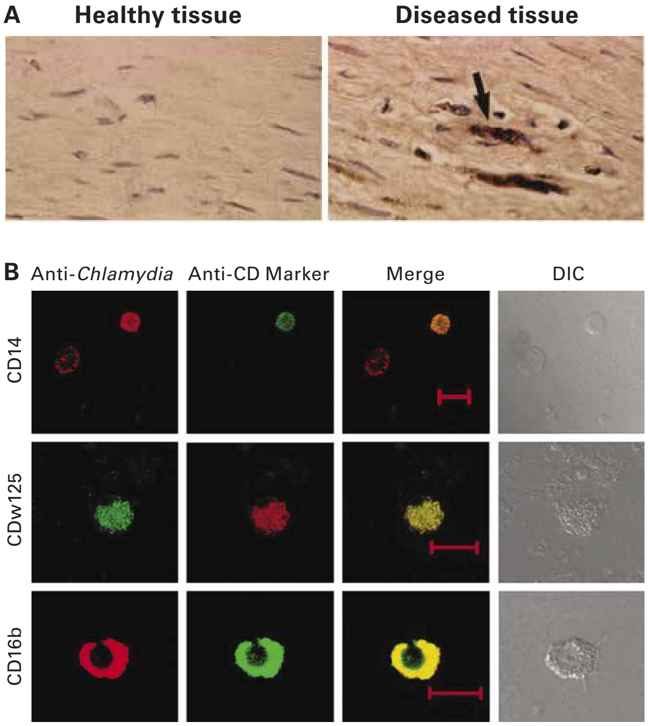


Figure 6-6. Immunohistochemistry and immunofluorescence microscopy to detect *Chlamydophila pneumoniae* in infected tissues. (A) Shown are microscopic images of immunohistochemical staining of coronary artery wall tissue using specific monoclonal antibodies against *C. pneumoniae* labeled with biotin and complexed with avidin-biotin-conjugated hydrogen peroxidase as a reporter enzyme. Left panel: normal healthy tissue section. Right panel: a diseased tissue section with an atherosclerotic plaque (arrow). Reprinted from Campbell LA, Kuo CC, Grayston JT. 1998. *Emerg Infect Dis* **4**:571–579, with permission. **(B)** Shown are micrographs of cells infected with *C. pneumoniae* and labeled with fluorescently labeled antibodies against *C. pneumoniae* and different cellular markers (CD14, CDw125, and CD16b). Cells infected with bacteria exhibit colocalization of fluorescent markers. Reprinted from Cirino F, Webley WV, West C, Croteau NL, Andrzejewski C Jr, Stuart ES. 2006. *BMC Infect Dis* **6**:23, with permission.

After washing away any unbound material, the pathogen is detected by applying the primary antibodies generated against the pathogen. The pathogen-specific antibodies directly conjugated to a colorimetric reporter enzyme, or the secondary antibodies conjugated to the reporter enzyme that recognize the primary antibodies, are then added. The colorimetric assay is then performed, as stated earlier.

Eliminate the Pathogen and Prevent or Cure the Disease

Even the use of a human volunteer to show that ingesting *H. pylori* could cause a mild inflammation of the stomach lining did not convince everyone of the *H. pylori*-ulcer connection. What finally convinced most scientists and physicians was the development of an antibiotic therapy that eliminated the bacteria and at the same time cured the disease (Box 6-3). In principle, information about a particular microbe should enable scientists to design effective therapeutic or preventive measures for eliminating the disease-causing microbe. In practice, successful prevention or treatment of the disease by therapeutic elimination of the disease-causing microbe is the ultimate test of a proposed microbe-disease connection. But it is important to point out that it is necessary to show that antibacterial therapy removes the microbe *and* cures the disease, eliminating the symptoms. This strategy of using antibiotics to cure disease as a way of testing the microbial origin of disease was not possible in Koch's time because antibiotics were not discovered until the 1930s. In a sense, however, around Koch's time there was other supporting evidence for this general approach of eliminating the pathogen to stop the spread of disease that helped convince skeptics that bacteria caused cholera.

John Snow (1813–1858), another early skeptic of the miasma theory, traced the source of a cholera outbreak in the Soho area of London in 1854 to a particular water well that

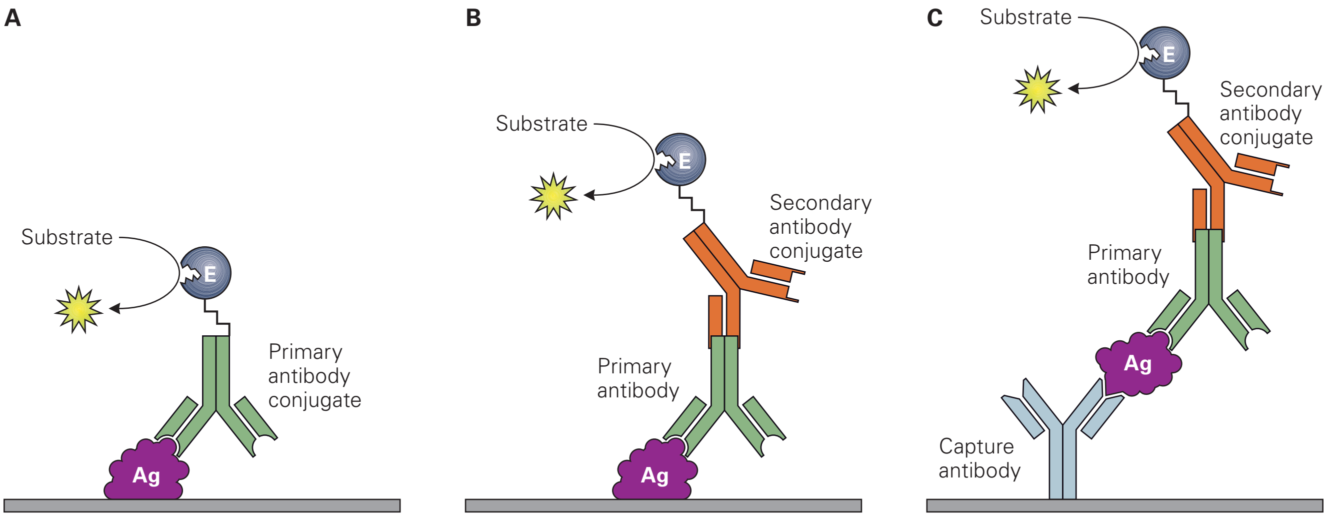


Figure 6-7. Enzyme-linked immunosorbent assays (ELISAs) for detection of pathogens in infected tissues. Pathogen antigens present in infected tissue or blood samples, or host antibodies against the pathogen antigens present in blood or serum, can be detected by various ELISA methods using antibodies generated against the pathogen: **(A)** Direct ELISA. **(B)** Indirect ELISA. **(C)** Capture or Sandwich ELISA.

Box 6-3.

Old Dogma versus New Model

Although the discovery of *Helicobacter pylori* is often believed to have occurred in the 1980s, evidence of Gram-negative bacteria in the stomachs of patients at autopsy has been reported since the late 19th century. Indeed, in the 1940s, New York City hospitals treated ulcers with antibiotics such as tetracycline, but by the 1950s antibiotics ceased to be used because scientists and doctors could not prove all of Koch's postulates for the involvement of bacteria in ulcer formation. For the next four decades, medical texts attributed peptic ulcers to too much stomach acid, stress, smoking, alcohol consumption, and genetic predisposition. The stress-acid ulcer theory gained a lot of credibility in the 1970s, when safe and effective agents that reduced gastric acid, such as histamine H_2-receptor blockers (e.g., Tagamet), were found to often heal or reduce painful ulcers. But when people stopped taking these blockers, the ulcers recurred, so typically these ulcer sufferers had to take those drugs for the rest of their lives. Consequently, these drugs became a very lucrative pharmaceutical business.

Well, in 1983, this dogma changed with the first published report by Robin Warren and Barry Marshall connecting *H. pylori* and chronic gastritis. Although it took more than 10 years (in 1994 there was finally an official report confirming the microbial source of peptic ulcers), it is now generally accepted that *H. pylori* is the primary causative agent of gastroduodenal inflammation and peptic ulcer disease, which is readily treatable and even curable with antibiotic treatment.

Comparison of treatment strategies for gastric ulcers

	OLD DOGMA	NEW MODEL
Cause	Excess stomach acid damages tissues, causes inflammation	*Helicobacter pylori* secretes toxins that cause inflammation, damages tissues
Treatment	Bland diet (no citrus or spicy foods, no alcohol or caffeine); histamine H_2-receptor blockers; surgery to remove ulcers	Antibiotic regimen (one to four weeks) of one or two antibiotics plus an antacid
Success	Ulcers recur if H_2-receptor blockers are discontinued	No recurrence after completion of antibiotic therapy
Cost	H_2-receptor blockers cost \$60 to \$100 per month; surgery costs up to \$18,000	One week of therapy costs less than \$200

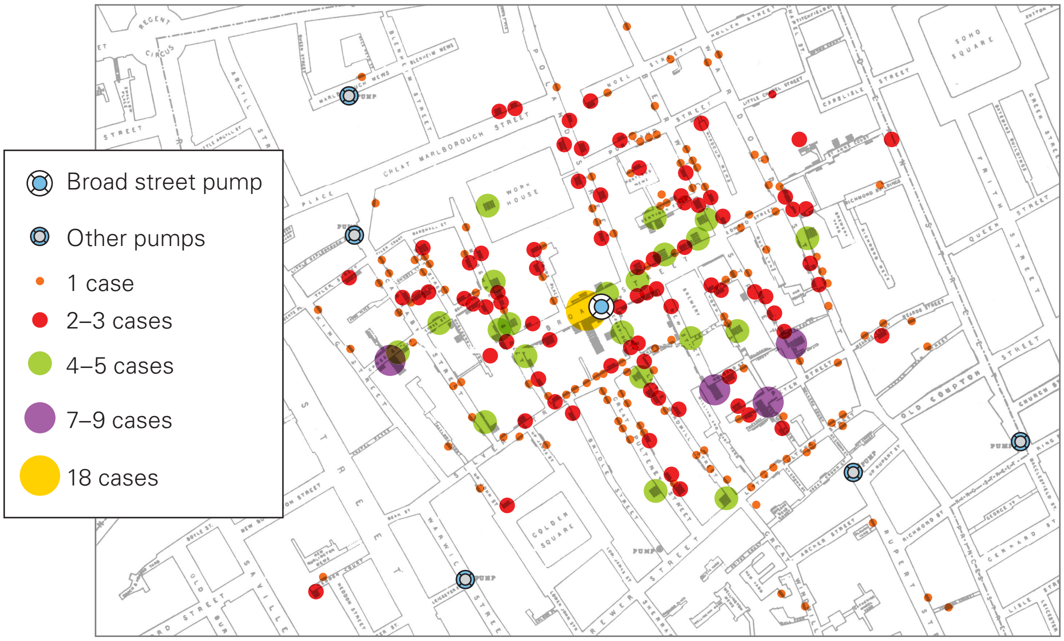


Figure 6-8. A city map illustrating the clusters of cholera cases in the 1854 London epidemic outbreak. John Snow used a dot distribution map to show the location of households affected by cholera and traced the source to a particular water pump. In his published article about the cholera outbreak, he used statistics to connect the quality of water from the pump source and the reported cholera cases. He also determined that the water from the source pump was coming from sewage-contaminated water from the Thames River.

was at the geographical center of the outbreak (Figure 6-8). His published work on the properties and the source of disease spread (**transmission**) of *Vibrio cholerae* helped establish the founding principles of modern **epidemiology**. In part through his work, public health officials were able to prevent the further spread of cholera by identifying and shutting off contaminated water sources and later by filtering or treating water with chlorine to eliminate *V. cholerae* from the water supply. Thus, by eliminating the disease-causing microbe or by preventing exposure to the microbe, the disease itself was eliminated or prevented, thereby strongly supporting the cause-and-effect relationship.

As another example, scientists working on *C. pneumoniae* had technically satisfied all four of Koch's postulates, provided that one accepts the rabbit model as a good model for human atherosclerotic disease. Nonetheless, there were still skeptics. The proponents of the link between *C. pneumoniae* and heart disease are likewise trying to find an antibiotic regimen that prevents or cures atherosclerosis. However, finding such a regimen has so far proven difficult. Antibiotics that kill a bacterium in the laboratory may not have the same effect in the human body because of the way the antibiotic is distributed and because of local conditions that may destroy the antibiotic. It took years to find the appropriate antibiotic combinations that finally subdued *H. pylori* because of the low pH of stomach contents that destroyed most antibiotics and required the development of protective drug formulations. But once a suitable formulation was found and demonstrated to be effective in most cases, successful treatment of gastric disease with antibiotics finally allowed for widespread acceptance of the hypothesis that *H. pylori* causes ulcers.

Results to date of clinical trials testing whether antibiotics can prevent or cure atherosclerosis have been mixed. Some show an effect and some do not. The cases in which antibiotic treatment was found not to have an effect have been used to argue against *C. pneumoniae* as the cause of atherosclerosis. This argument was also used in the early days of the search for an antibiotic treatment of ulcers. To complicate things even more, besides inhibiting bacterial growth, many antibiotics have the propitious side effect of reducing inflammation. Since inflammation is known to exacerbate the atherosclerotic condition, this might suggest that the apparent positive effects of antibiotics in clinical trials could reflect either killing of the bacterium or reduction of inflammation.

Along similar lines, prevention of a disease by **vaccination** that induces immunity against a specific pathogen can also be used to prevent or lessen disease and again provide evidence of cause and effect, particularly if the immunized individual is subsequently exposed to the pathogen and does not display symptoms of the disease. Vaccination has been used repeatedly to demonstrate bacterial as well as other microbial causes of infectious diseases for which a microbe was clearly implicated. But it is now also being used as a way of showing causality for diseases whose etiology has not yet been clearly defined.

One recent example of the use of this vaccination approach to establish the microbial origin of disease is aimed at preventing cervical cancer. The major risk factor for cervical cancer is infection by human papillomavirus (HPV). There is much excitement about several HPV vaccines that have been available since 2006. So far, the vaccine has shown high efficacy against genital warts and four common strains of HPV that are associated with cervical cancer. If the excellent track record for preventing cervical cancer continues to hold long-term, this will add to the proof that HPV causes cervical cancer.

Comparative Infectious Disease Causation

Another area in which researchers continue to struggle with microbial origin of disease causation is the case of infections caused by stealth pathogens, so called because of the persistent nature of their infections that go on for protracted periods of time (months to years) before they are detected and symptoms appear. The host immune system fails to clear the stealth pathogen, and the chronically infected individual is often in a carrier state without overt disease symptoms. How can a direct connection between these microbes and the long-term disease sequelae be made? One proposal that has been made is to study the comparative biological and pathological characteristics of the infections caused by the putative stealth pathogen across multiple mammalian species.

The genus *Bartonella* is an example of a stealth pathogen for which disease causation has been difficult to establish. These intracellular bacteria invade and reside in erythrocytes and endothelial cells, causing long-lasting intravascular infections in reservoir hosts and chronic relapsing bacteremias in cases of opportunistic infections in nonreservoir hosts. To satisfy Koch's postulates, it has been proposed by researchers who study *Bartonella* infections that disease causation can be established if the same bacterium can be isolated from or detected by modern molecular technologies during a naturally occurring infection in multiple different mammalian genera in which the disease pathology is the same. For example, there is now a substantial body of evidence that supports the ability of members of the genus *Bartonella*, including *B. henselae, B. quintana, B. bacilliformis*, and *B. vinsonii,* to cause naturally occurring chronic intravascular infections that are believed to lead to subsequent vascular endothelial cell tumors in immunocompromised humans, dogs, cats, and other animals with similar disease pathologies. Since infections with *Bartonella* are often asymptomatic, it might be too late to treat the persistent infection with antibiotics to prevent later disease sequelae such as cancer. Instead, to confirm a causal relationship between *Bartonella* infection and disease, it might be necessary to develop a vaccine against *Bartonella* species that prevents chronic infections by this stealth pathogen and subsequent cancer.

As should be evident from the above discussion, it can be very hard to be absolutely sure, without any doubt, that microbe X causes disease Y. There is enough room for error in each of Koch's postulates to make absolute certainty a virtual impossibility in some cases. Moreover, as we have seen in the case of diseases like syphilis, failure to satisfy one or more of Koch's postulates does not necessarily disprove a connection between a microbe and a disease. In the end, the preponderance of evidence (as the lawyers would say) is what convinces scientists that there is a cause-and-effect relationship.

The Microbiota Shift Disease Problem

Koch's Postulates and Pathogenic Microbial Communities

If it is difficult to prove a connection between one microbe and a disease, what does one do about polymicrobial diseases? A number of diseases, such as periodontal disease (associated with gum erosion and tooth loss) and bacterial vaginosis (associated with higher risk for preterm birth and increased susceptibility to STDs), appear to be caused by shifts in the composition of bacterial populations of the body (the **microbiota**) that contain hundreds of species. Scientists working in this area have tried to construct postulates similar to Koch's postulates in which "pure culture" is replaced by "a shift from one specified population to another." Experimental evidence so far suggests that a **microbial population shift** often favors certain compositions of microbes that are not normally found in microbial communities from healthy individuals **(microbial dysbiosis)**. In these cases, not just one microbe is responsible for the resulting disease, but rather the composition of an ensemble of microbes is associated with the dysbiosis (the pathogenic **microbial community**) that triggers host inflammatory responses, which then cause tissue damage and disease symptoms. For these polymicrobial diseases, the third postulate presents a challenge because it is nontrivial to produce specific population shifts in laboratory animals. Nevertheless, there are several areas of research nowadays in which this is being actively pursued with some success.

For microbial shift diseases, such as inflammatory bowel disease, periodontal disease, and bacterial vaginosis, the use of selective antibiotics or vaccines may turn out to be of great importance in providing supporting evidence equivalent to Koch's third postulate for pure cultures. Here, the question then becomes: if the original composition of the bacterial population can be restored after antibiotic treatment, is the disease prevented? This area will be a challenge for future microbiologists, especially if more such diseases are shown to be microbiota shift diseases.

Keystone Pathogens and Microbial Shift Diseases

Periodontal disease is a microbial biofilm-associated inflammatory disease of the tissues surrounding the teeth (**periodontium**). This is a chronic condition that can lead to **gingivitis** (swelling and bleeding of the gums), progressive

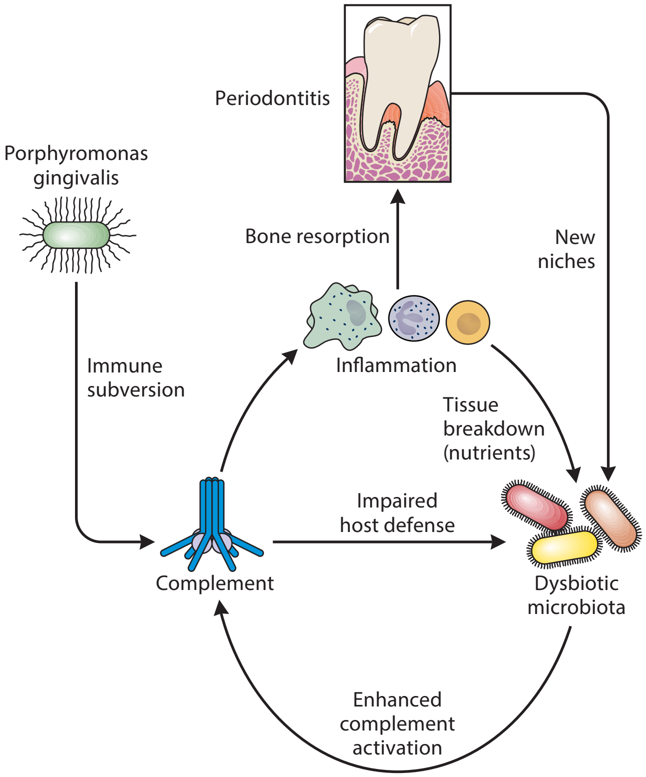


Figure 6-9. *Porphyromonas gingivalis*-induced dysbiosis of microbiota and periodontal disease. *P. gingivalis* subverts the host's complement system to cause inflammation while also impairing host defenses through the inhibition of leukocyte killing. This alters the normally benign composition of the commensal microbiota, leading to microbial dysbiosis and enhanced inflammation, which in turn exacerbates colonization and overgrowth of more pathogenic bacteria, causing more inflammation, tissue damage, bone resorption, and periodontal disease. Adapted from Hajishengallis G, Darveau RP, Curtis MA. 2012. *Nat Rev Microbiol* **10:**717–725, with permission.

loss of the bone around the teeth and dental decay (**caries**), and, in severe cases, tooth loss. Even since Koch's time, it has been generally agreed that the microbes attached to the surface of teeth, which form dental plaques that calcify and cause inflammation, are the source of the inflammation that leads to **periodontitis**. The biofilm that forms **dental plaque** consists of a complex community of over 700 different bacterial species. Experimental evidence so far suggests that a microbial population shift that favors certain Gram-negative anaerobes is responsible for the initiation of the microbial dysbiosis (Figure 6-9). Regardless of the etiology of the microbial dysbiosis, the ensuing host immunologic response is the primary factor that results in inflammation and contributes to the tissue destruction and bone loss characteristic of periodontal disease.

The initially proposed model was that periodontal disease was caused by a succession of microbial shifts in the biofilm, starting with a pathogenic microbial community, the so-called orange complex comprised of *Fusobacterium nucleatum* and one or more *Prevotella* species. The resulting dysbiosis causes inflammation, which in turn causes another microbial shift to another more pathogenic microbial community, the so-called red complex comprised of *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola*. Modern molecular detection technologies, such as PCR and DNA sequencing, have consistently demonstrated a higher prevalence of these bacteria in subsequent development of periodontitis compared to healthy dental tissues. However, there are some problems with this model. For one, studies have shown that the putative periodontal pathogens *P. gingivalis* and *T. forsythia* are also frequently isolated from healthy tissues. In another study, an entirely different set of bacteria was associated with health and disease; namely, the Gram-negative bacterium *Veillonella* was found to be associated with healthy tissue, while the Gram-positive anaerobic bacterium *Filifactor alocis* was associated with periodontal disease.

This model has now been further challenged with some recent findings from a study aimed at elucidating the role of *P. gingivalis* in periodontitis by using a mouse model of periodontal bone loss. In this study, the researchers used three different groups of mice: (1) wild-type mice that had normal commensal bacteria; (2) **germ-free (GF)** mice that were completely sterile of bacteria; and (3) **specific pathogen-free (SPF)** mice that had normal commensal bacteria but were free of a specific pathogen, in this case *P. gingivalis*. As expected, no bone loss was observed in control SPF or GF mice, while, like the wild-type mice, the *P. gingivalis*-treated SPF mice developed bone loss. But, surprisingly, no bone loss was observed for the *P. gingivalis*-treated GF mice. These findings indicated that not only was *P. gingivalis* required for development of periodontal disease, but also the presence of a microbial community was required for the disease process. Moreover, the researchers found that *P. gingivalis* did not have to be present in high concentrations to mediate its effect. They further found that this effect was mediated by a particular virulence factor produced by the bacteria, namely a cysteine protease called gingipain that cleaves the complement C5 protein. The resulting C5a activates the C5a receptor, thereby increasing inflammatory responses but at the same time preventing bacterial killing by leukocytes, such as polymorphonuclear leukocytes (PMNs), NKs, and macrophages. The resulting inflammation and bacterial overgrowth causes the observed shift in microbiota (Figures 6-9 and 6-10).

These findings suggest that a single low-abundance bacterium can instigate changes in normally benign microbiota that result in pathogenic outcomes, such as conversion to a microbial dysbiosis that triggers host inflammation, tissue damage, and development of periodontitis. The researchers argued that *P. gingivalis* acts as a **keystone pathogen** based on its disproportionately large impact on a microbial com-

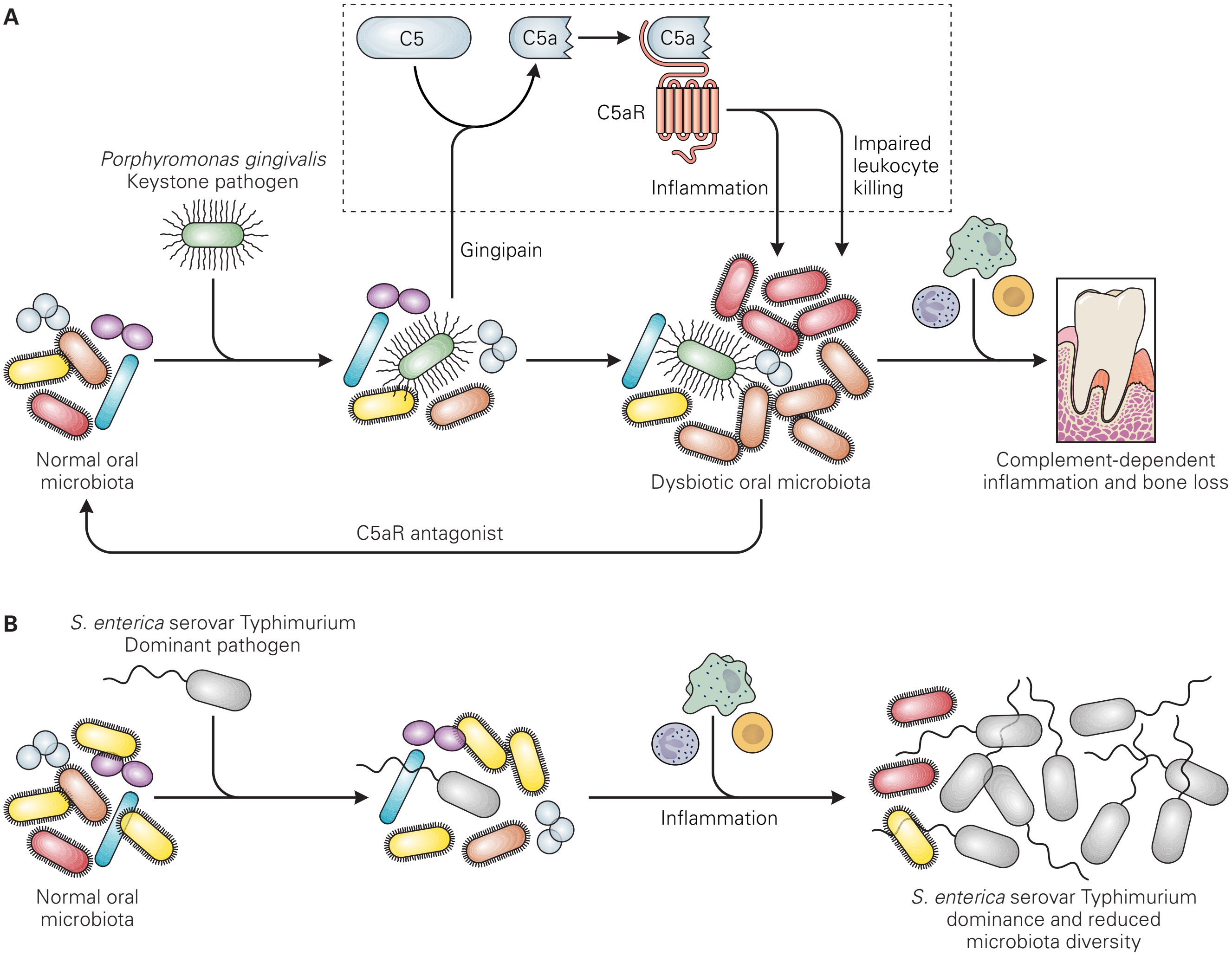

A
C5
C5a
C5a
C5aR
Inflammation
Impaired leukocyte killing
Porphyromonas gingivalis
Keystone pathogen
Gingipain
Normal oral microbiota
Dysbiotic oral microbiota
Complement-dependent inflammation and bone loss
C5aR antagonist
B
S. enterica serovar Typhimurium
Dominant pathogen
Inflammation
Normal oral microbiota
S. enterica serovar Typhimurium dominance and reduced microbiota diversity

Figure 6-10. Disease progression via a keystone pathogen versus a dominant pathogen. (A) *P. gingivalis* acts as a keystone pathogen by modulating the host immune response to increase inflammation while also impairing leukocyte killing. *P. gingivalis* secretes a cysteine protease (gingipain) that cleaves complement protein C5 into C5a, which binds to the C5a receptor (C5aR), which in turn regulates inflammatory responses and impairs leukocyte killing of bacteria. This leads to overgrowth and changes in the composition of the microbiota, leading to microbial dysbiosis and more inflammation and disease progression. Removal of the C5aR or treatment with a C5aR antagonist drug allowed the recovery of the normal composition of microbiota and prevented bone loss. **(B)** A dominant pathogen such as *Salmonella enterica* serovar Typhimurium directly induces inflammation and microbial dysbiosis by overgrowing and outcompeting or even killing off the normal microbiota. Adapted from Hajishengallis G, Darveau RP, Curtis MA. 2012. *Nat Rev Microbiol* **10**:717–725, with permission.

munity with respect to its relatively low abundance (Figure 6-10). This is in stark contrast to what happens with most bacterial pathogens, which cause inflammatory disease due to outgrowing, outcompeting, and/or outright killing the normally protective commensal microbiota, thereby becoming the dominant bacterial species present and inducing acute, overt symptoms of disease.

Molecular Koch's Postulates

Just as microbiologists in the second half of the 19th century struggled with the question of how to prove that a particular bacterium caused a particular disease, microbiologists in the second half of the 20th century struggled with the question of how to prove that a particular gene or set of genes from a pathogen contributed to virulence. If Koch were alive today, he would no doubt be flattered by the fact that the initial attempts to answer this question used his postulates as a guide, but he would not be surprised to see these postulates argued just as hotly as the postulates he put forward. On the other hand, Pasteur, more than Koch, anticipated and would appreciate the new understanding of the adaptability and evolution of bacteria during the disease process.

There have been several versions of molecular Koch's postulates proposed, but most of them read as follows. First, the gene (or its product) should be found only in strains of bacteria that cause the disease or phenotype and not in bacteria that are avirulent or do not show the phenotype. Second, the gene should be isolated by cloning and sequencing. Third, disrupting the gene in a virulent strain or a strain exhibiting the phenotype should show a loss of function, as evidenced by a measurable reduction or attenuation of its virulence or disappearance of its phenotype. Alternatively, introducing the cloned gene into an avirulent strain should render the strain virulent or should confer the phenotype on a strain lacking that phenotype. Finally, it should be demonstrated that the pathogenicity or phenotype is restored when the gene is reintroduced into the mutant bacterium and that the gene is expressed by the bacterium when it is in an animal or human volunteer at some point during the infectious process.

Today, this view of genes conferring virulence or phenotypic traits seems overly simplistic, but at the time attempts to establish a set of criteria for what would be called a "virulence gene" or a "phenotype gene" were instrumental in getting the field off to a running start. This molecular version of Koch's postulates also did a service to the scientific field because almost immediately it generated dissatisfaction with such a simple view and fueled discussions that have led to a more sophisticated (but perhaps still not entirely correct) view of genes, their role in disease (see the following section), and their outcome in terms of virulence and genotype or phenotype (discussed in later chapters).

Concepts of Disease

Varieties of Human-Microbe Interactions

The early view of infectious diseases, implicit in Koch's postulates, was that there were microbes capable of causing disease and that exposure to them inevitably caused the disease. Already in Koch's time, however, this simplistic description of infectious diseases was being questioned. Scientists realized that not all people who drank water containing *V. cholerae* developed cholera. Not all people exposed to someone with tuberculosis developed symptomatic disease, even though it could be shown that they were infected with the bacterium. Similarly, not all people who harbor *H. pylori* develop gastric ulcers. Clearly, all humans are not equal in their response to an infectious microbe.

As scientists worked with disease-causing microbes, they further discovered that just as there are differences in susceptibility from person to person, there is also variation among different strains of the same bacterial species. Sometimes, a bacterium that had been grown too long in laboratory medium lost the ability to cause infection. Or different isolates of what appeared to be the same bacterium, even by comparison of the 16S rRNA genes (as described in chapter 5), differed in their ability to cause disease. Through the years, a greater appreciation for the complexity of the interaction between microbes and humans has emerged.

Currently, scientists view the infection process as a multifaceted interaction between the microorganism and the human body. This interaction may result in clearance of the microbe from the body, asymptomatic carriage of the microbe, or the development of symptoms years later. For example, infection with *T. pallidum*, which has so far not been cultivated in laboratory medium, manifests with four stages of the disease syphilis. How the symptoms appear for diagnosis of syphilis and the choice of modern molecular techniques used for establishing disease causation depend on the disease stage (see Box 6-4). The outcome of a human-microbe encounter depends on the infected person's genetic makeup, health status, immune system and other defenses against disease, as well as the traits of the infecting strain of the microbe. Finally, as described in the first chapter, ecology and environment contribute to infections. Diseases come and go depending on ecological and environmental factors, including human activities.

Views of the Human-Microbe Interaction

Although everyone agrees that the interactions between microbes and the human body are complex, there are differences of opinion about how these interactions should be understood. Perhaps the most widely held view is that disease-causing bacteria evolved specifically to cause human disease. A second view, which has gained more adherents lately, is that disease-causing bacteria are actually trying to achieve an equilibrium with humans that does not result in disease, and that disease symptoms result when this equilibrium does not develop or has not developed yet because the interaction is still at an early stage. Adherents of this view point to the fact that in many diseases, the number of people who develop serious infections is far smaller than the number of people who carry the bacteria without developing any symptoms. A third view is that humans are more often than not accidental hosts of some bacteria that may be able to cause human disease but have actually evolved to occupy some other niche, either out in the environment or in another host that serves as a reservoir. In this view, bacteria entering the human body trigger an immune response that in turn causes the bacteria to react by activating stress responses, producing disease-causing virulence factors and symptoms in the process.

Probably each of these views is correct for a subset of diseases. For example, a bacterium that causes disease only in humans, has no external reservoir, and causes symptoms in virtually all infected people fits the first view, whereas a bacterium that causes an asymptomatic carrier state in most infected humans may fit the second view. A bacterium that spends most of its time outside the human body and only occasionally causes human disease may fit more closely with the third view. As we shall see in later chapters, the varieties

Box 6-4.
Satisfying Koch's Postulates for a Clinical Case of Syphilis

Infection with the spirochete *Treponema pallidum* is believed to be the causative agent of the sexually transmitted disease syphilis, yet this bacterium has never been cultivated in the laboratory and does not cause human-like disease in animals, even though it will grow in live rabbit testicles. Certain antibiotics, such as penicillin, will eliminate the bacteria from rabbits, and antibiotic treatment cures syphilis infection and disease symptoms in humans. The problem with syphilis is that it has four disease stages, each with different symptoms:

1° stage syphilis—A single painless lesion (chancre) containing replicating bacteria is observed in 70% of cases (see Figure 6-4A). Bacteria can be detected using dark-field microscopy (see Figure 6-4B) or immunofluorescence microscopy (see Figure 6-4C). Treatment with antibiotics will clear the infection.

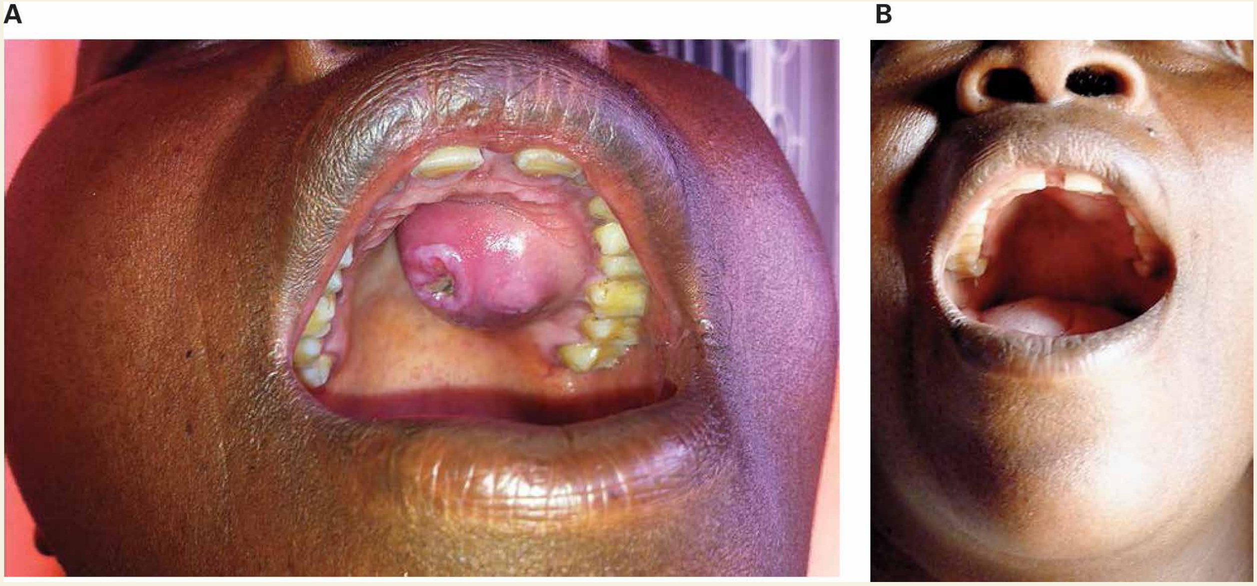
2° stage syphilis—Without treatment, a diffuse skin rash on the palm of hands and soles of feet and spreading throughout the body appears one to six months later and lasts for two to six weeks, but eventually clears.

Latency—During this stage of the disease, which can last for many years, there are no overt symptoms of disease, and the bacteria are difficult to detect in the body. Antibodies generated against *T. pallidum* can be detected in serum using ELISA.

3° stage of syphilis—This stage appears years later in various forms of disease manifestations: gumma (large granulomas, masses, or lesions caused by reactivation of infection), or central nervous system, eye, or heart problems (due to autoimmunity or hypersensitivity reactions). Antibiotic treatment will slowly clear the infection and gumma but not always other sequelae.

In cases of 3° syphilis, misdiagnosis is an unfortunately common event. One such recent clinical case of syphilitic gumma was reported from an outreach clinic in rural Uganda, where a 45-year-old woman presented with a year-long history of a progressively enlarging ulcerated mass on the upper palate of her mouth (see panel A of figure below). The mass was initially painless, but became increasingly painful, causing difficulty in speaking and swallowing. She had no history of syphilitic symptoms or treatment, and the clinic doctors diagnosed the mass as cancer. She was in the midst of selling her home to pay for surgical resection to remove the tumor when another team of doctors double-checked the lesions on the mass for pathology and discovered the spirochetes under microscopic analysis. Subsequent ELISAs confirmed the presence of *T. pallidum,* and 3° syphilis with oral gumma was diagnosed. The patient received injections with antibiotics (penicillin G), and two months later the mass and symptoms (panel B in figure below) were completely resolved. Happily, she was able to keep her home.

Source:
Cherniak W, Silverman M. 2014. Images in clinical medicine: syphilitic gumma. *N Engl J Med* **371:**667, with permission.



of human-bacterium interactions are so numerous and distinct that there may be no one model that fits all diseases. This problem is compounded further by the recent discovery of the complexity and dynamics of the host microbiomes (see chapter 5) and the possible roles this association plays in modulating health and susceptibility to diseases, including bacterial infections.

Virulence as a Complex Phenomenon

In the early days of modern pathogenesis research, things seemed much simpler than they do today. This is probably because the first diseases to be studied in depth at the molecular level were diseases like diphtheria that were caused by bacteria that produced a single toxic protein (diphtheria toxin) responsible for the symptoms of the disease. A toxin is clearly a virulence factor. As more complex diseases were probed, however, it became obvious that in some cases a large number of diverse virulence factors were involved in the ability of a bacterium to cause disease.

An experimental definition of a virulence factor has been that loss of the factor by the bacterium results in a decrease in its ability to cause disease. This seems pretty straightforward, but what does one do about cases in which the loss of two factors reduces the pathogenicity of the bacterium, but loss of each trait separately does not? Or about a trait that is clearly a virulence factor in one bacterium but is not a virulence factor in another bacterium? Then there are the cases in which the loss of the ability to synthesize an amino acid makes the bacterium unable to cause disease. Can the biosynthesis of an amino acid, a trait normally considered to be a routine part of the microbe's basic physiology (generally considered a housekeeping factor), also be considered a virulence factor? Even more confusing are the cases in which the loss of a trait makes a bacterium more virulent.

Throughout this book you will encounter examples of scientists grappling with this problem of how to define a virulence factor. Either the current definition of a virulence factor is inadequate or the entire concept of a virulence factor, which implies that a small number of discrete traits make the difference between the ability to cause disease and the inability to cause disease, needs to be rethought. Rather than critique the definition of virulence factor and early versions of molecular Koch's postulates to identify virulence factors, it makes more sense to consider the ways in which modern molecular methods have provided new ways to investigate how bacteria cause disease.

To cope with the multifactorial nature of virulence, many modern molecular methods have been designed that do not just focus on one or a few genes, but seek mutations that affect some general feature of pathogenesis, such as the ability to survive inside a phagocyte, the expression of a set of genes in an animal, or survival in the animal. Examples of this are approaches that seek genes expressed only in the animal or that eliminate the ability of a bacterium to survive in the animal when they are missing. More recently, scientists are turning to comparative genomics and other next-generation DNA-sequencing technologies (see chapter 5) to identify genes or sets of genes involved in the pathogenesis of bacterial infection. All of these methods generally identify numerous genes as being important in the animal. These approaches, as well as others, will be described in more detail in later chapters.

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Questions

1. Three views of bacterial pathogens have been proposed. In one view, disease-causing bacteria evolved specifically to cause disease in humans. (They're out to get you!) In the second view, bacteria evolved to colonize certain sites in a certain animal and cause disease when they do not establish equilibrium with their host. (They're out to dine at your expense!) In the third view, humans were often accidental hosts for bacteria whose traits were not designed for colonizing or infecting humans. (They're lost and not happy about it!) What sort of traits in a bacterial pathogen would convince you that one of these views was the best explanation for a particular disease?
2. Why are scientists so reluctant to let go of Koch's postulates even though they often chafe—as Koch did—under their restrictions? Why are Koch's postulates, with all of their problems, still as relevant today as they were when Koch first proposed them?
3. Could you prove cause and effect using something like Koch's postulates if you could not cultivate the organism you suspect is causing the disease? What type of approach would you use?
4. We mentioned the possibility that some diseases might be caused by shifts in the microbiota of a site on the human body. Along these lines, some oral microbiologists have pointed to an association between gingivitis (gum disease), caused by microbes found in dental plaques, and cardiovascular disease. Could you formulate Koch's postulates for such a disease? If so, how would you set up an experiment to satisfy these postulates?
5. Animal models are often used to satisfy Koch's postulates. Yet mice and other laboratory animals sometimes react differently than humans to infectious agents. For example, *Salmonella* Typhi*,* the cause of typhoid fever in humans, a very serious disease, does not cause disease in mice. In other cases the bacteria localize differently in humans and mice. *Salmonella* Typhimurium causes a fatal bloodstream infection in mice but only causes diarrhea in most humans. What features of an animal model need to be evaluated before using such a model to satisfy Koch's postulates?
6. Are there certain disease cases where it might be allowable to use human volunteers as experimental subjects in order to satisfy Koch's postulates? What are those instances?

Solving Problems in Bacterial Pathogenesis

1. A group of researchers at the USDA isolate a new bacterium from the lungs and lymph nodes of several young horses that became ill and died at a local stable. Prior to death, symptoms included disorientation and loss of motor function, so the researchers suspected central nervous system involvement, which was confirmed by the observation of brain lesions in the dead animals. Based on 16S rRNA comparison, the bacterium is distantly related to *Neisseria meningitidis*, and they subsequently named it *Neisseria equiniae*. The researchers believe that *N. equiniae* may be responsible for the brain lesions observed in a small percentage of older horses that die of apparent dementia. What four criteria must be satisfied in order for the researchers to prove that the brain lesions in these older horses are caused by *N. equiniae*? Provide at least three modern molecular experiments that could be performed to satisfy these criteria.

2. You have just received an urgent call from the Centers for Disease Control and Prevention (CDC) to consult on the following case of an unusual disease outbreak that they have been investigating. A new Gram-positive bacterium related to *Listeria monocytogenes* was isolated from an outbreak of food poisoning in Wisconsin due to contaminated cheese that appears to cause painful gastritis and, in about half of the exposed individuals, sudden onset of bleeding ulcers, followed by death from toxic shock within two to three days. Upon biopsy of the infected individuals, it was found that the bacteria were growing on the surface of epithelial cells lining the gastric pit of the stomach. Autopsy of individuals who died showed that bacteria were found only in the stomach and not in any of the other body organs. The researchers at the CDC have subsequently determined that, like *Listeria monocytogenes*, this new species of *Listeria* invades epithelial cells in tissue culture. Which, if any, of Koch's postulates have been satisfied for the involvement of this new *Listeria* strain with gastritis, bleeding ulcers, and toxic shock? What additional measures could the researchers take to help satisfy Koch's postulates?

3. *Porphyromonas gingivalis*, a Gram-negative, non–spore-forming, heterodiploid, anaerobic, black-pigmented bacterium, is widely considered to be an important etiological agent of periodontal disease because of its strong correlation with active disease process and abscess formation in animal infection models. Therapy for severe periodontal disease usually involves scraping to remove dental

plaque and debridement (surgical removal of affected tissue and abscesses) in combination with a two-week regimen of anaerobe-specific metronidazole and/or a three- to five-week regimen of tetracycline. A mouse abscess model of infection has been developed for *P. gingivalis*, in which the bacteria are injected into the skin of the animal and allowed to form an abscess over the course of a week. The lesion site where the abscess is forming can be surgically removed and plated on blood agar plates and incubated in an anaerobic chamber to grow colonies after about five days of incubation. There is an emerging paradigm shift in our current understanding of the causes of coronary heart disease from a purely hereditary and nutritional causation to possibly having an infectious etiology. This shift comes from recent epidemiological studies that have implicated a correlation between periodontal disease and coronary heart disease. There is a recent report that *P. gingivalis* can invade oral epithelial tissues during severe disease and exacerbate the inflammatory responses. Inflammatory responses have been linked to atherosclerosis (plaque formation in arteries) that could lead to heart disease. In support of this, another recent report found that *P. gingivalis* could also invade human primary coronary artery endothelial cells in tissue culture. Considering all of the previous information, which of Koch's postulates, if any, have been satisfied so far for *P. gingivalis* involvement in heart disease? Be sure to state your rationale. Provide at least four different modern molecular experiments that could be performed to help satisfy Koch's postulates for *P. gingivalis* involvement in heart disease.

4. In 1976, the Bellevue-Stratford Hotel in downtown Philadelphia hosted a convention of members of the American Foreign Legion. During the convention, 221 legionnaires developed a new form of pneumonia and 34 people died. Infectious disease specialists working for the CDC were sent to Philadelphia to identify the cause of this new, mysterious illness, which not surprisingly was subsequently named Legionnaires' disease. Using Koch's postulates, list the steps that those specialists had to take to complete their assignment. Briefly explain the significance of each step. These specialists understood that it is not always possible to follow Koch's postulates to the letter. As they embarked on their quest to identify the causative agent of this new illness, list at least two problems that they might have faced in attempting to fulfill Koch's postulates.

5. In 1984, Barry Marshall took a unique approach to fulfilling Koch's postulates. He drank a culture of the bacterium *Helicobacter pylori* and developed a mild case of gastritis, although another volunteer developed a more severe case of gastritis. He and his mentor, Robin Warren, won the Nobel Prize in Physiology or Medicine in 2005 for their discovery of the role of *H. pylori* in the pathogenesis of gastritis and peptic (stomach and duodenal) ulcers. Knowing that 80% of peptic ulcer disease cases are associated with *H. pylori* infection, briefly describe how each of Koch's postulates are fulfilled for the link of *H. pylori* with peptic ulcer disease. What modern approaches can be taken to help satisfy Koch's postulates for this case?

7

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Acquiring New Virulence Traits by Horizontal Gene Transfer

Mechanisms of Genetic Change and Diversification

Spontaneous Mutation

Phase Variation

Antigenic Variation

Horizontal Gene Transfer: Mobile Genetic Elements

Natural Transformation

Conjugation: Plasmids and Transposons

Phage Transduction

Control of Horizontal Gene Transfer

Toxin-Antitoxin Systems—Retaining the Goods

Restriction-Modification Systems—Bacterial Innate Immunity from Foreign DNA

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CHAPTER 7

Mechanisms of Genetic Modification and Exchange

ROLE IN PATHOGEN EVOLUTION

Imagine kissing someone and acquiring from that person a whole new chromosome, which gives you features of the person you kissed. Sound like a far-fetched science fiction scenario? For humans it is, but it is actually quite commonplace among microbes. Bacteria routinely undergo a process called conjugation, in which two bacterial cells engage in close contact with each other as one transfers segments of DNA into the other. These DNA segments can be extremely large, even hundreds of kilobases, accounting for as much as one-third of some bacterial chromosomes. Of course, the DNA segments that are typically transferred are much smaller than this, but most still carry multiple genes. This process is one of a group of processes collectively referred to as horizontal gene transfer. Incredibly enough, most of the time, these large additions of DNA are well tolerated and can grant the bacteria powerful new traits such as resistance to antibiotics, the ability to utilize new nutrient sources, or new abilities to cause disease. Additionally, many of these bacterial gene transfers occur across species and genus lines. It might be pure science fiction when Peter Parker became Spiderman after being bitten by a spider, but that is essentially what happens all the time in the bacterial world.

Adapt or Perish

Acquiring New Virulence Traits by Horizontal Gene Transfer

The ability of a bacterium to respond to new selective pressures, to survive adverse environmental conditions, or to exploit new environments that it encounters depends on its ability to evolve through modification of gene function via mutation or acquisition of new genes through **horizontal gene transfer (HGT)**. Until recently, it was thought that changes to a microbe's virulence properties would arise by slow processes involving point mutations, gene duplications, gene deletions, or chromosomal rearrangements, and that adaptive changes would occur largely through antigenic or phase variation (Table 7-1). The prevailing view was that in order to maintain or amplify these mutations once they arose, it was necessary to have a strong, persistent selective pressure, which could only be applied by specific exposure to the host environment. New evidence, particularly from genome sequencing analyses of many bacteria, has shown that high frequency exchange of large DNA sequences between different species and even different genera by HGT contributes significantly to genome variability and accounts for a much more rapid evolution of pathogens than was previously thought. Moreover, DNA transferred by HGT is often stably maintained in the absence of selection.

Likewise, it was commonly believed that disease-causing microbes evolved over long periods of time, through complex interactions between the microbe and its host. However, increasing evidence suggests that the evolution of genes encoding most bacterial toxins and other virulence factors involves HGT mediated by plasmids, transposons, bacteriophages, and other transmissible DNA elements (Table 7-1). HGT allows for rapid acquisition of genes encoding antibiotic resistance, toxins, and other virulence factors (see chapters 11 and 12). These acquired genes can facilitate survival in an animal or human host by helping the bacteria evade or suppress the host's immune system, enable growth on varied nutrient resources, enhance survival under various environmental conditions, and permit dissemination within the host, as well as facilitate dissemination into the external environment or transmission to new hosts. All of these traits provide mechanisms for a bacterium's continued survival under adverse conditions inside or outside the host. HGT also allows for these traits to arise and evolve separately before coming together in a single bacterium to enhance, or even impart, virulence. See Box 7-1 for an interesting twist on the implications of HGT and evolution.

Table 7-1. Mechanisms of Genetic Change and Diversification

SLOW PROCESSES	RAPID PROCESSES
Point mutation	Phase variation
Nucleotide change	Promoter inversion
Nucleotide insertion	Transcriptional blockage
Nucleotide deletion	Slipped-strand synthesis
Gene duplication	Antigenic variation
Gene deletion	Gene shuffling
Chromosomal rearrangement	Gene conversion
Inversion	HGT
Intragenic recombination	Intergenic recombination
	Transformation
	Plasmid
	Conjugation
	Plasmid
	Transposon
	Transduction
	Phage
	Generalized
	Specialized

Mechanisms of Genetic Change and Diversification

Spontaneous Mutation

Although the focus of this chapter is on HGT, it is worthwhile to review other mechanisms of evolutionary change that occur by mutation, since these processes are constantly occurring in the background as genetic material is transferred between organisms. Although these mutations are generally less impactful individually than larger gene acquisitions occurring during HGT, their ubiquitous nature can still have a profound impact on bacterial cellular processes and virulence.

The first class of spontaneous mutations is single nucleotide exchanges or replacements (**single nucleotide polymorphisms** or **SNPs**), which occur at rates ranging from 10^{-7} to 10^{-10} per nucleotide per generation, depending on the bacterium. The second class of spontaneous mutations is recombination events that encompass various forms of DNA duplication, insertions/deletions (**indels**), or rearrangements (Figure 7-1) and can occur at a rate of 10^{-4} to 10^{-6} mutations per cell per generation. Genetic changes affecting both single base pairs or large DNA segments result in heritable changes that can lead to genes or gene products with altered biological activity or regulation, which in turn can result in effects on growth, fitness, and virulence. For example, tandem duplication of a region of the bacterial chromosome can result in two copies of a gene encoding a regulatory protein, and this increase in gene dosage will in turn increase the cellular amount of the regulatory protein, which may affect the expression of the genes it controls. Typically, when comparing the genomes of closely related species, the order or arrangement (**synteny**) of homolo-

Box 7-1.
War of the Worlds—Revisited

In H. G. Wells's classic science fiction thriller *War of the Worlds,* lethal infection by terrestrial microorganisms saved humankind from annihilation by an extraterrestrial army of killer Tripods. The alien invaders, who unlike us had not evolved alongside these microbes over millennia, had no natural immunity against these ordinary Earth microbes and thus became mortality ill upon exposure to us and our environment. Could the converse occur? Is it possible that extraterrestrial microorganisms introduced to Earth might be capable of causing human disease?

Astrobiologists are taking this possibility seriously and are concerned about how safe it is to bring back samples from our space exploits. Indeed, NASA has even created a special position entitled "Planetary Protection Officer," whose task is to protect Earth from extraterrestrial contamination. This person is responsible for determining whether extraterrestrial soil samples collected during space expeditions to the moon, Mars, or other planets pose a potential biohazard to Earth's inhabitants.

It was previously believed that harmful microbes could not evolve without a human or animal host environment to provide a selective pressure for increased virulence. However, the acquisition of large pieces of DNA by HGT provides a means for gaining virulence traits in the absence of strong selection. And, with the finding that considerable HGT can occur outside the human or animal environment, it is conceivable that genetic exchange of material by bacteria in the external environment might pose unknown risks, particularly if those sources of genetic material evolve in unusual environments, such as those of other planets.

Source:

Mancinelli RL. 2003. Planetary protection and the search for life beneath the surface of Mars. *Adv Space Res* **31:**103–107.

gous genes in operons is conserved, but modular exchanges of large DNA sequence segments through chromosomal rearrangements, inversions, tandem gene duplications, deletions, or other recombination events can also occur, contributing significantly to the genetic diversity among bacterial populations.

When certain bacterial populations are stressed by nutrient deprivation and extended stationary phase conditions, a small subpopulation of cells becomes hypermutable. That is to say, a small fraction of the population will start acquiring spontaneous mutations at an increased rate relative to the rest of the bacterial population. This hypermutability phenotype appears to involve the expression of an error-prone DNA polymerase that introduces frequent mutations in the genome as the chromosome is replicated. Many of these stress-induced mutations end up being deleterious for the cell that carries them, but any bacteria with mutations that enable them to cope with the imposed stress will be able to propagate and become the dominant members of the population. This type of stress-induced mutagenesis has now been observed in many different bacterial species. As new, less expensive, next-generation sequencing technologies emerge, researchers will be able to explore how these mutations contribute to the long-term evolution of new traits.

Phase Variation

Changes in the expression of important virulence proteins that occur at relatively high frequency compared to spontaneous mutations are referred to as **phase variation**. This process can occur by several different mechanisms. One of the first examples of phase variation reported involves changing the composition of flagella (motility structures) of *Salmonella* species. Proteins that make up flagella are highly antigenic because they are present on the surface of the bacteria. It is advantageous for a bacterium to be able to periodically change its flagellar proteins to avoid elimination by the host's adaptive immune response. In the 1940s, it was observed that some *Salmonella* strains could switch from making flagella with the type H1 flagellin protein to flagella with the type H2 flagellin protein, and back again, at frequencies of about 10^{-4} per cell, which is considerably higher than normal mutation rates. This switching between the expression of different versions of the genes, encoding flagellin, is caused by a **DNA invertase** (called Hin), which promotes **inversion of sequences** upstream of the H2 flagellin gene. Figure 7-2 depicts the steps involved in the *Salmonella* flagellin H1/H2 phase-variation process. The invertible sequence region consists of the invertase gene, its promoter, and an additional promoter that can regulate downstream genes. When the invertible sequence is in one orientation, the second promoter transcribes the H2 gene and a downstream repressor of the H1 gene promoter. When the invertible sequence is in the other orientation, the H2 gene and the repressor will no longer be transcribed and the H1 gene will be expressed instead. Phase variation (or phenotypic switching) has also been found to play an integral role in the formation of diverse phenotypes within microbial communities that make up biofilms.

Another example of phase variation through promoter inversion occurs in *Mycoplasma* species. These bacteria are obligate intracellular pathogens that are able to constantly alter the expression of their surface antigens, allowing them

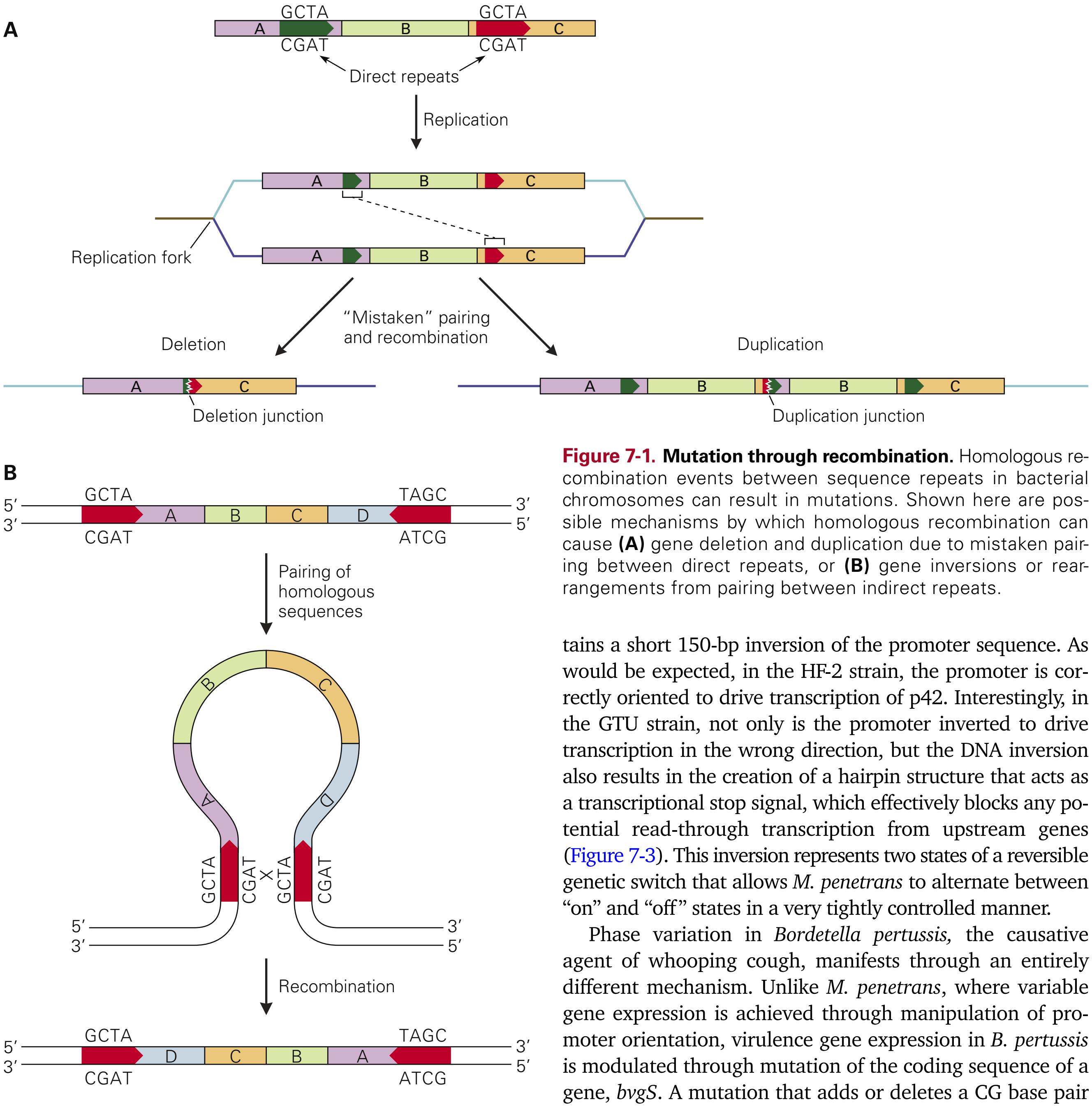

A
GCTA
CGAT
GCTA
CGAT
A
B
C
Direct repeats
Replication
A
B
C
Replication fork
"Mistaken" pairing and recombination
Deletion
Duplication
A
C
Deletion junction
A
B
B
C
Duplication junction
B
5′
3′
GCTA
CGAT
A
B
C
D
TAGC
ATCG
3′
5′
Pairing of homologous sequences
B
C
A
D
GCTA
CGAT
X
GCTA
CGAT
5′
3′
3′
5′
Recombination
5′
3′
GCTA
CGAT
D
C
B
A
TAGC
ATCG
3′
5′

Figure 7-1. Mutation through recombination. Homologous recombination events between sequence repeats in bacterial chromosomes can result in mutations. Shown here are possible mechanisms by which homologous recombination can cause **(A)** gene deletion and duplication due to mistaken pairing between direct repeats, or **(B)** gene inversions or rearrangements from pairing between indirect repeats.

to colonize the host even in the presence of an adaptive immune response. Most of the variable surface antigens of *Mycoplasma* species are lipoproteins, whose genes undergo frequent phase variation (on/off) and size variation (short/long) during infection. Insight into this mechanism comes from a comparison of the antigenic lipoproteins p42 in two closely related strains of *Mycoplasma penetrans*, strains HF-2 and GTU. The p42 lipoprotein is produced in the HF-2 strain but not in the GTU strain. The DNA sequence encoding p42 is identical between the two strains; however, the upstream regulatory region of this gene contains a short 150-bp inversion of the promoter sequence. As would be expected, in the HF-2 strain, the promoter is correctly oriented to drive transcription of p42. Interestingly, in the GTU strain, not only is the promoter inverted to drive transcription in the wrong direction, but the DNA inversion also results in the creation of a hairpin structure that acts as a transcriptional stop signal, which effectively blocks any potential read-through transcription from upstream genes (Figure 7-3). This inversion represents two states of a reversible genetic switch that allows *M. penetrans* to alternate between "on" and "off" states in a very tightly controlled manner.

Phase variation in *Bordetella pertussis,* the causative agent of whooping cough, manifests through an entirely different mechanism. Unlike *M. penetrans*, where variable gene expression is achieved through manipulation of promoter orientation, virulence gene expression in *B. pertussis* is modulated through mutation of the coding sequence of a gene, *bvgS*. A mutation that adds or deletes a CG base pair in a sequence of six consecutive Cs causes a frameshift in the BvgS protein that regulates virulence (Figure 7-4). This type of phase variation results from **slipped-strand misrepair** (also sometimes called **slipped-strand synthesis**) at sites of repeated DNA sequences (e.g., ATG-$[C]_n$-gene or ATG-$[CTCTT]_n$-gene), which can lead to different protein sequences or truncations from frameshifts. Slipped-strand misrepair has emerged as a common mechanism of phase variation in numerous pathogens, such as Opa and Opc (Opa-like) proteins in *Neisseria* species, which undergo reversible phase variation at a rate of 10^{-3} to 10^{-4} per cell per generation. Opa proteins are integral outer membrane proteins expressed in most meningococcal and all gonococcal

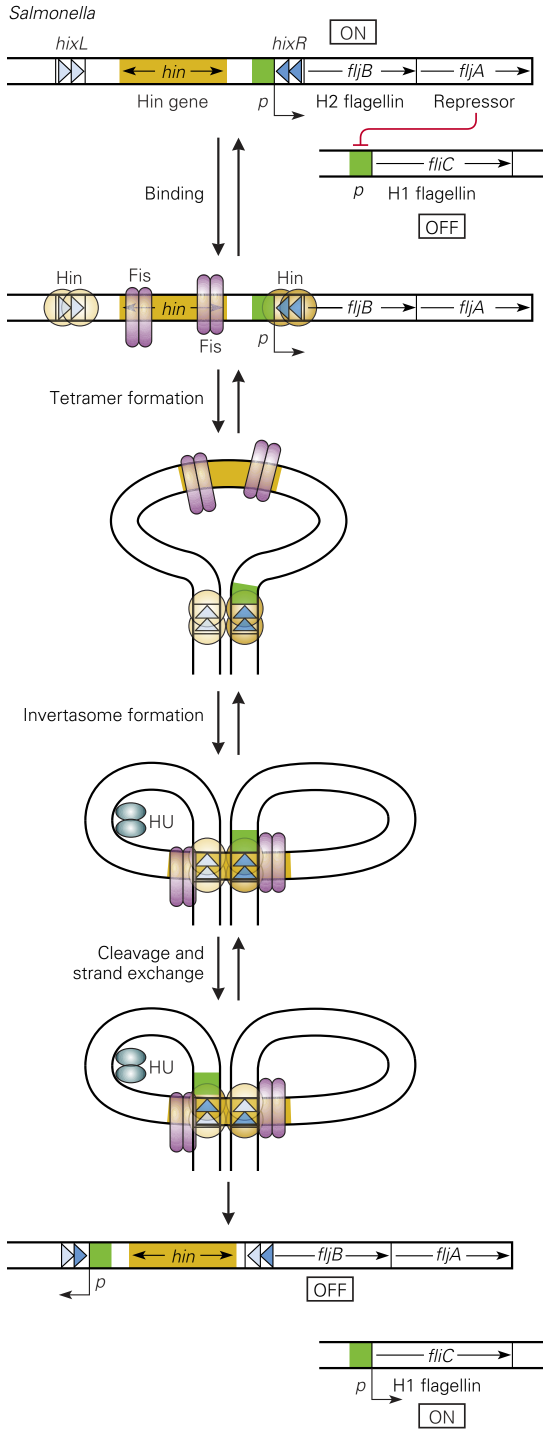


Figure 7-2. Phase variation in *Salmonella* by Hin invertase-mediated inversion. The Hin invertase inverts a sequence upstream of the gene for H2 flagellin (*fljB*) and a repressor of the H1 flagellin gene (*fliC*). The protein HU helps bend the DNA to facilitate formation of the invertasome. In one orientation, the H2 flagellin gene and the *fljA* repressor gene are transcribed from the promoter. In the other orientation, these two genes are not transcribed, and so the repressor no longer represses transcription of the H1 flagellin gene and H1 is expressed. The Hin invertase is constitutively expressed under its own promoter. Adapted from Nanassy OZ, Hughes KT. 1998. *Genetics* **149:**1649–1663, with permission.

strains and mediate intimate adherence and invasion during infection. *Neisseria meningitidis* strains have 3 or 4 *opa* genes, and *Neisseria gonorrhoeae* strains have 8 to 13 *opa* genes, any of which may be on or off at different stages during infection. All *opa* genes contain 5′ tandem repeats of [CTCTT]$_n$ in the signal peptide coding region of the gene that cause high frequency of slipped-strand frameshifts and loss or gain of expression (Figure 7-5). *Neisseria* species also produce a distinct type of membrane surface sugar-lipid molecule called **lipooligosaccharide (LOS)**. LOS differs from lipopolysaccharide (LPS) in that it has short, highly branched sugar units attached to the lipid core and no repeating O-antigen units. LOS is a very toxic molecule because of its strong activation of the innate immune system, resulting in the local inflammation and tissue damage that is characteristic of a *Neisseria* infection. The surface structure of LOS is highly variable because many of the genes encode enzymes (**glycosyltransferases**) that add sugars to LOS. These genes have a tract of poly-Gs in the 5′ region of the gene's sequence. Slipped-strand misrepair occurs in these regions at high frequency, resulting in variable expression of these glycosyltransferase enzymes. Additionally, LOS can be sialylated during different stages of infection, which also contributes to serum resistance. Sialic acid groups are covalently attached to galactose residues on the LOS by **sialyltransferase** enzymes, which also undergo slipped-strand misrepair phase variation. Depending on which glycosyltransferases and sialyltransferases are being expressed at any given time, the LOS will have different surface structures (Figure 7-6).

Antigenic Variation

Pathogenic microbes can also avoid the host immune system by changing their surface antigens through gene-shuffling events (**antigenic variation**). The pili of *N. gonorrhoeae,* which are responsible for attaching to host epithelial cells, are highly antigenic and undergo both phase and antigenic variation by recombination between different pilin genes. In addition to the major pilin gene *pilE*, which has a promoter, each genome contains multiple (10 to 20) silent copies of the pilin gene *pilS*, which lacks a promoter. The *pilE* and *pilS* genes share regions that are highly conserved, but they also have regions that differ significantly, the so-called hypervariable regions. Recombination events between the conserved regions of any of the *pilS* genes and *pilE* result in a new version of the *pilE* gene (Figure 7-7), and hence a new pilin protein on the surface, which is not recognized by existing antibodies in the host. Pilin recombination in *Neisseria* requires a functional RecA protein, as well as RecO and RecQ (RecF-like recombination), and is a type of **gene conversion**, since it is nonreciprocal (occurs only in one direction).

In *Neisseria*, phase and antigenic variation are so prevalent and important for survival in the host that they are considered the major mechanisms of *Neisseria* pathogenesis. Because of this type of variation, the host's immune system

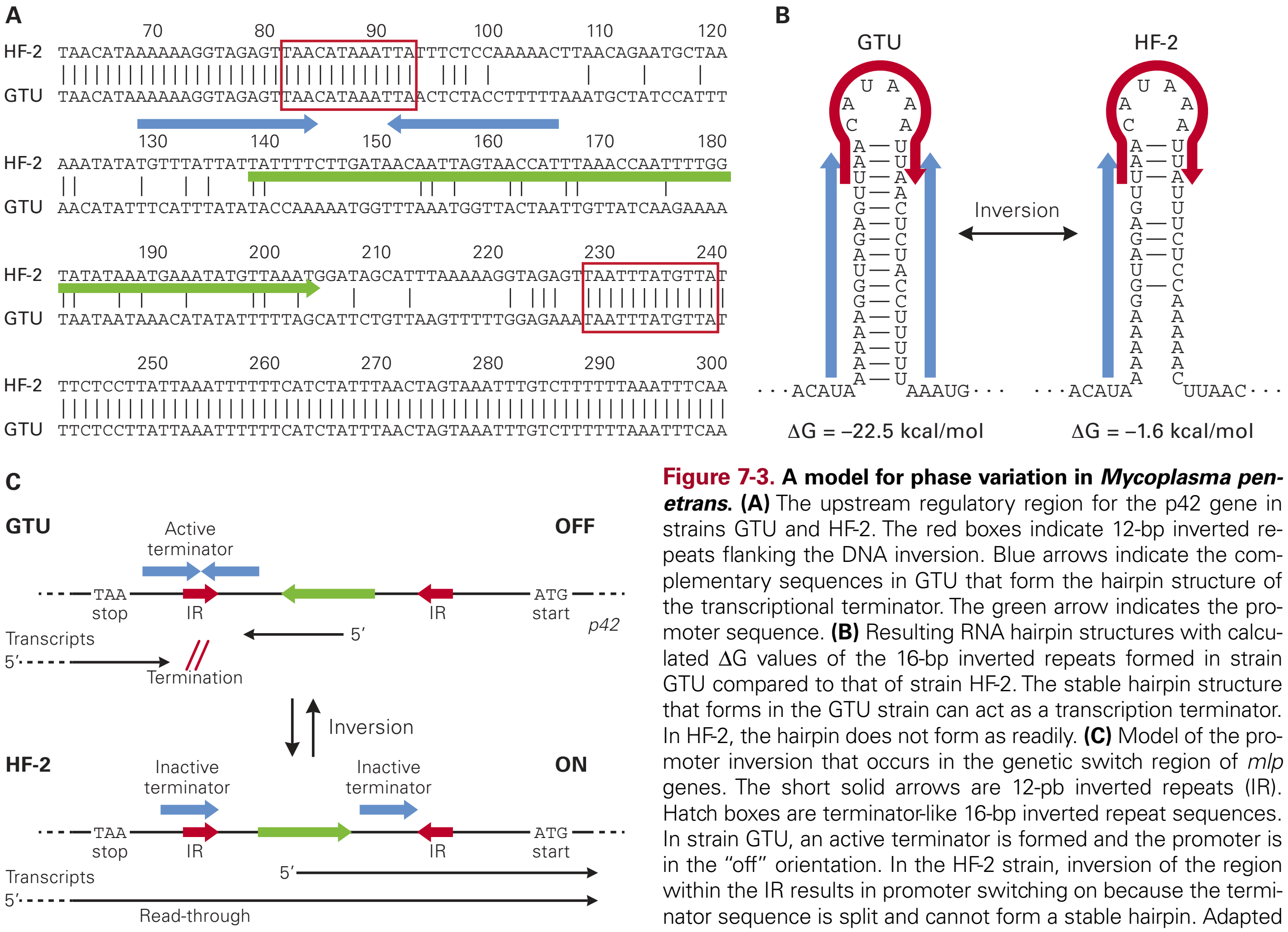

A
70
80
90
100
110
120
HF-2 TAACATAAAAAAGGTAGAGTTAACATAAATTATTTCTCCAAAAACTTAACAGAATGCTAA
GTU TAACATAAAAAAGGTAGAGTTAACATAAATTAACTCTACCTTTTTAAATGCTATCCATTT
130
140
150
160
170
180
HF-2 AAATATATGTTTATTATTATTTTCTTGATAACAATTAGTAACCATTTAAACCAATTTTGG
GTU AACATATTTCATTTATATACCAAAAATGGTTTAAATGGTTACTAATTGTTATCAAGAAAA
190
200
210
220
230
240
HF-2 TATATAAATGAAATATGTTAAATGGATAGCATTTAAAAAGGTAGAGTTAATTTATGTTAT
GTU TAATAATAAACATATATTTTTAGCATTCTGTTAAGTTTTTGGAGAAATAATTTATGTTAT
250
260
270
280
290
300
HF-2 TTCTCCTTATTAAATTTTTTCATCTATTTAACTAGTAAATTTGTCTTTTTTAAATTTCAA
GTU TTCTCCTTATTAAATTTTTTCATCTATTTAACTAGTAAATTTGTCTTTTTTAAATTTCAA
B
GTU
HF-2
Inversion
· · · ACAUA
AAAUG · · ·
· · · ACAUA
UUAAC · · ·
ΔG = −22.5 kcal/mol
ΔG = −1.6 kcal/mol
C
GTU
Active terminator
OFF
TAA stop
IR
IR
ATG start
p42
Transcripts
5′
5′
Termination
Inversion
HF-2
Inactive terminator
Inactive terminator
ON
TAA stop
IR
IR
ATG start
Transcripts
5′
5′
Read-through

Figure 7-3. A model for phase variation in *Mycoplasma penetrans*. (A) The upstream regulatory region for the p42 gene in strains GTU and HF-2. The red boxes indicate 12-bp inverted repeats flanking the DNA inversion. Blue arrows indicate the complementary sequences in GTU that form the hairpin structure of the transcriptional terminator. The green arrow indicates the promoter sequence. **(B)** Resulting RNA hairpin structures with calculated ΔG values of the 16-bp inverted repeats formed in strain GTU compared to that of strain HF-2. The stable hairpin structure that forms in the GTU strain can act as a transcription terminator. In HF-2, the hairpin does not form as readily. **(C)** Model of the promoter inversion that occurs in the genetic switch region of *mlp* genes. The short solid arrows are 12-pb inverted repeats (IR). Hatch boxes are terminator-like 16-bp inverted repeat sequences. In strain GTU, an active terminator is formed and the promoter is in the "off" orientation. In the HF-2 strain, inversion of the region within the IR results in promoter switching on because the terminator sequence is split and cannot form a stable hairpin. Adapted from Horino A, Sasaki Y, Sasaki T, Kenri T. 2003. *J Bacteriol* **185:**231–242, with permission.

cannot keep up. Repeated infections with *Neisseria* are common, and indeed, variation often occurs within a single infected individual. There is usually a vigorous antibody response, yet genetic variability in pili and other surface antigens always occurs in a significant fraction of the bacterial population at any given time. Although pressure from the immune response may allow a particular pilin variant to become the dominant form in a population, once antibodies are made against that variant, it is rendered obsolete, and bacteria expressing one of the minor variants then take over and become the next dominant form in the population. Since millions of possible pilin variants can arise from phase and antigenic variation, no effective antibody response can be mounted against *N. gonorrhoeae*, and repeated infections occur.

Although *Neisseria* pilin recombination can occur at a significant frequency within a single bacterium (**intragenic recombination**), most of the variation needed for host immune evasion results from recombination between the genes from different bacterial cells. DNA containing *pilS* is released when the donor cell lyses. Recipient cells pick up this extracellular DNA, allowing the *pilS* from a donor cell to recombine with the *pilE* of the recipient cell (**intergenic recombination**). This intergenic recombination is possible because of two properties of *Neisseria*. First, *Neisseria* bacteria frequently undergo autolysis to release their genomic DNA. Second, *Neisseria* cells are naturally transformable (see next section) in that they are able to take up the extracellular DNA and incorporate it into their genomes through homologous recombination. By undergoing intergenic recombination, *Neisseria* are able to attain even greater antigenic variation than might occur through intragenic recombination alone.

Horizontal Gene Transfer: Mobile Genetic Elements

Natural Transformation

Bacteria can exchange DNA in three ways: transformation, conjugation, or transduction. The first mechanism of gene exchange in bacteria to be discovered was **natural transformation**: DNA from a **donor cell** is released into the environment and is then taken up by a **recipient cell.** The resulting progeny are called **transformants**. Most bacteria do not take up DNA efficiently unless they have a little help from an external force (such as a scientist) in the form of exposure to certain chemical or electrical treatments that make them more permeable (bacteria that are capable of taking up DNA are said to be **competent**). The field of molecular biology is extremely reliant on a scientist's ability to introduce recombinant DNA into chemically competent or electrocompetent bacteria.

There are, however, a number of Gram-negative and Gram-positive bacteria, including some notable pathogens (*Haemophilus influenzae, H. parainfluenzae, N. gonorrhoeae, N. meningitidis, Campylobacter jejuni, Helicobacter pylori, Streptococcus pneumoniae, Vibrio cholerae, Legionella pneumophila, Acinetobacter baylyi,* and *Aggregatibacter actinomycetemcomitans*), which are or can become **naturally transformable (naturally competent)**. Natural competence allows for the uptake of DNA from the environment and incorporation of the homologous DNA into the chromosome. Frederick Griffith (1879-1941) in 1928 performed the first experiment demonstrating natural transformation in the pathogen *S. pneumoniae*. Griffith observed that *S. pneumoniae* came in two varieties, a pathogenic one (S strain) that formed smooth colonies on agar plates (now known to occur due to secretion of a polysaccharide capsule) and a nonpathogenic one (R strain) that formed rough colonies. In his classic experiment (Figure 7-8), Griffith found that if he injected mice with the S strain, they became sick and died, but if he first heat-killed the S strain before injection or if he injected the R strain, then the mice did not die. Surprisingly, if he mixed the live R strain with the heat-killed S strain before injection, then the mice died. He also found that the bacteria recovered from the dead mice formed smooth colonies on agar plates. That is, the R strain had converted into an S strain just by mixing with the dead components of the S strain. He concluded that there must be a "transforming principle" in the sample of dead S strain bacteria that transferred the pathogenic trait to the live R strain bacteria. It was not until sixteen years later in 1944, that the "transforming principle" in Griffith's experiment was determined to be DNA by Oswald Avery (1877–1955), Colin MacLeod (1909-1972), and Maclyn McCarty (1911–2005).

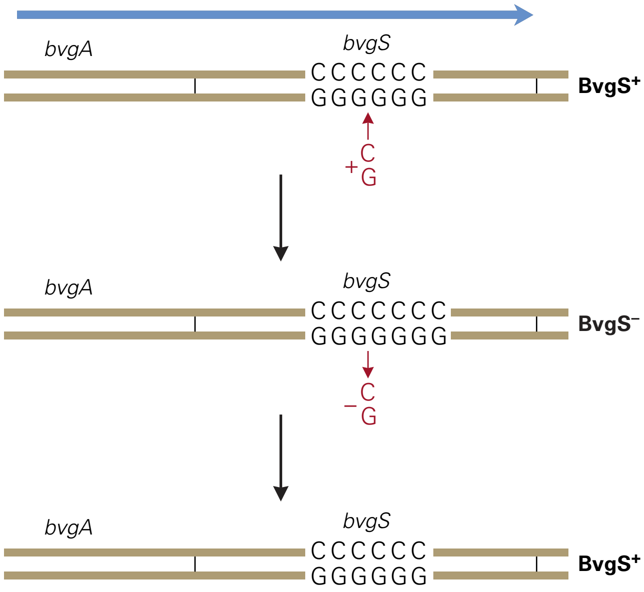


Figure 7-4. Phase variation in *Bordetella* by slipped-strand misrepair. *B. pertussis* toxin and other virulence factor genes are controlled by a two-component regulatory system, consisting of the BvgS and BvgA proteins. Phase variation results from a frameshift mutation in a region of the *bvgS* gene containing a string of Cs. Based on data from Stibitz S, Aaronson W, Monack D, Falkow S. 1989. *Nature* **338:**266–269.

Many naturally competent bacteria have short (10 to 12 base pairs) **DNA-uptake sequences (DUS)** scattered throughout their genomes that serve as recognition sites for binding and uptake of the DNA into a competent cell. Some bacteria (e.g., *N. gonorrhoeae* and *H. influenzae*) have specific receptors that recognize their own DUS sequences (5′-GCCGTCTCAA-3′ and 5′-AAGTGCGGTCA-3′, respectively) and allow them to take up DNA only from their own

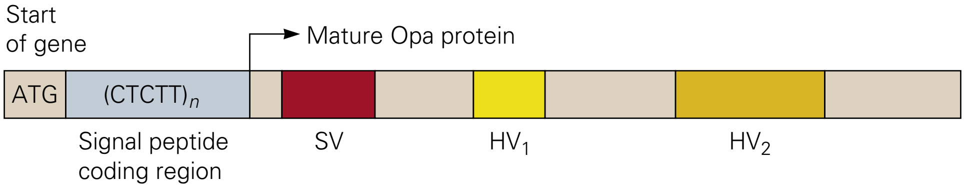


Figure 7-5. All *Neisseria* outer membrane protein (*opa*) genes contain 5′ tandem repeats in the signal peptide coding region of the gene. Slipped-strand misrepair occurs at the [CTCTT]$_n$ repeats in the N terminus of the *opa* gene, resulting in alteration of reading frame and loss of expression of the protein. In addition, each *opa* gene contains semivariable (SV) and hypervariable (HV) regions. Data from Murphy GL, Connell TD, Barritt DS, Koomey M, Cannon JG. 1989. *Cell* **56:**539–547.

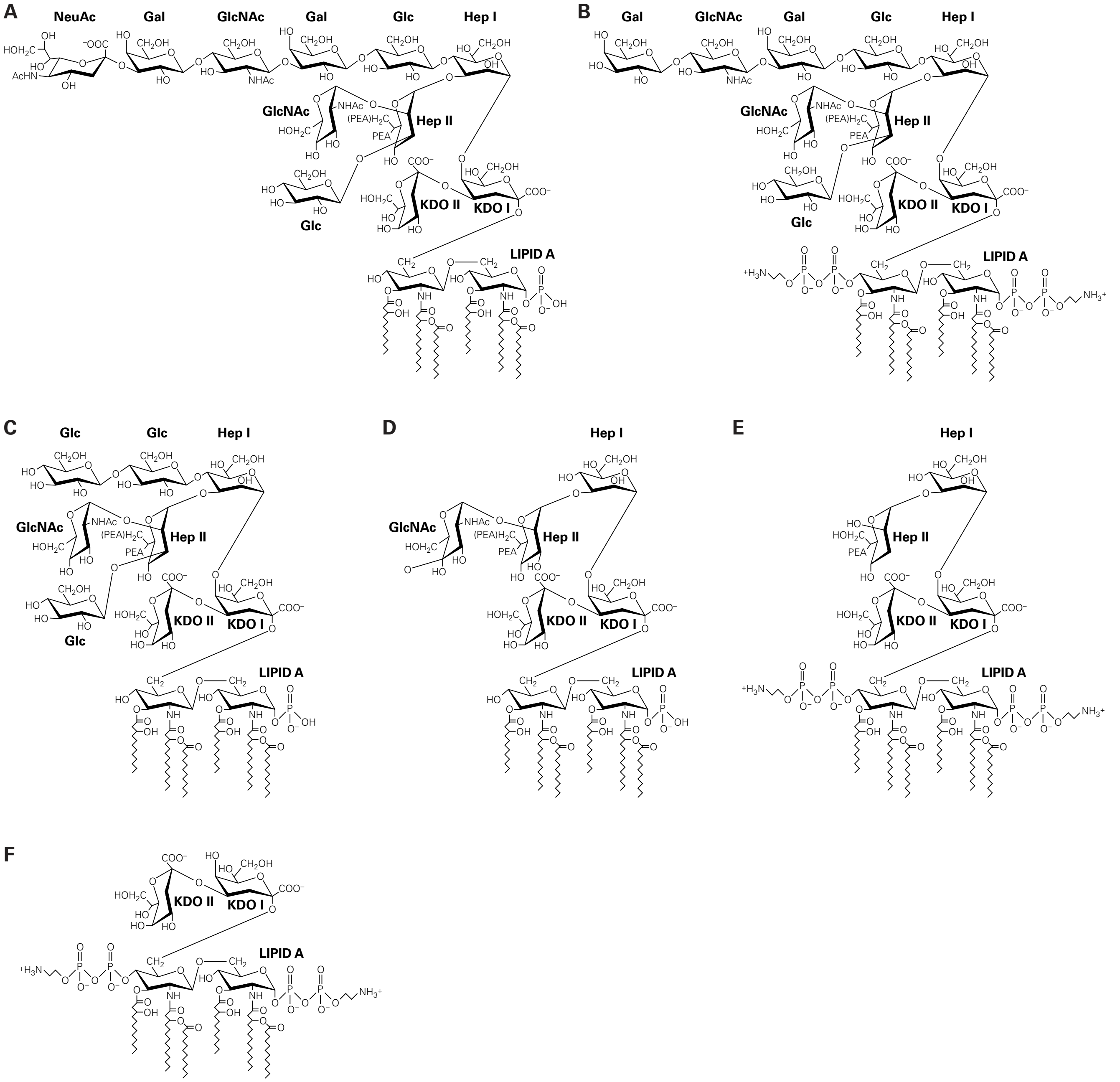

A
NeuAc
Gal
GlcNAc
Gal
Glc
Hep I
GlcNAc
Hep II
Glc
KDO II
KDO I
LIPID A
B
Gal
GlcNAc
Gal
Glc
Hep I
GlcNAc
Hep II
Glc
KDO II
KDO I
LIPID A
C
Glc
Glc
Hep I
GlcNAc
Hep II
Glc
KDO II
KDO I
LIPID A
D
Hep I
GlcNAc
Hep II
KDO II
KDO I
LIPID A
E
Hep I
Hep II
KDO II
KDO I
LIPID A
F
KDO II
KDO I
LIPID A

Figure 7-6. Antigenic variation leads to different lipooligosaccharide (LOS) structures in *Neisseria meningitidis* serogroup B strain NMB. Shown is the predominant LOS structure of *N. meningitidis* serogroup B wild type strain NMB and **(A)** mutant strains with LOS variants **(B-F)** generated by mutation of genes in the biosynthetic pathway of different components of the LOS molecule. Reproduced from Kahler CM, Martin LE, Shih GC, Rahman MM, Carlson RW, Stephens DS. 1998. *Infect Immun* **66:**5939–5947, with permission.

species. In contrast, other bacteria, such as *Bacillus subtilis* and *S. pneumoniae*, are able to take up any DNA, but only homologous DNA sequences are recombined into the chromosomes of recipient cells. In many bacteria, competence is highly regulated. This regulation often involves bacteria receiving a quorum-sensing signal during growth, as well as other environmental factors, such as acidity (pH below 7.0). For example, the quorum-sensing signal for *S. pneumoniae* is a small, linear peptide (called competence stimulatory peptide). Bacteria that sense these competence signals initiate a complex cascade of gene expression that produces the competence proteins needed to uptake DNA and recombine homologous DNA into the recipient cell's chromosome. In addition, some bacterial species, such as *V. cholerae* and

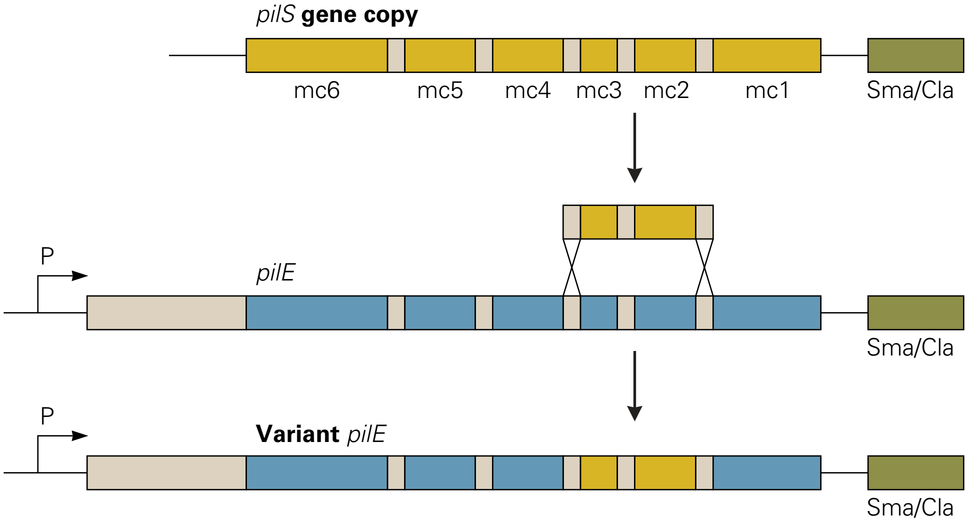


Figure 7-7. Antigenic variation in *Neisseria* by gene conversion. The pilin genes of *N. gonorrhoeae* can undergo nonreciprocal homologous recombination events between the expressed *pilE* locus and different silent copies of the *pilS* gene at other loci. The major conserved regions of *pilE* and *pilS* are represented by the shaded gray boxes, and the variable sequences are represented by either blue boxes for *pilE* or yellow boxes for *pilS*. At the 3′ end of all pilin loci is a conserved DNA sequence, called the Sma/Cla (S/C) repeat, which has sequence similarity to several recombinase-binding sites and is involved in efficient pilin recombination. Adapted from Hill SA, Davies JK. 2009. *FEMS Microbiol Rev* **33:**521–530, with permission.

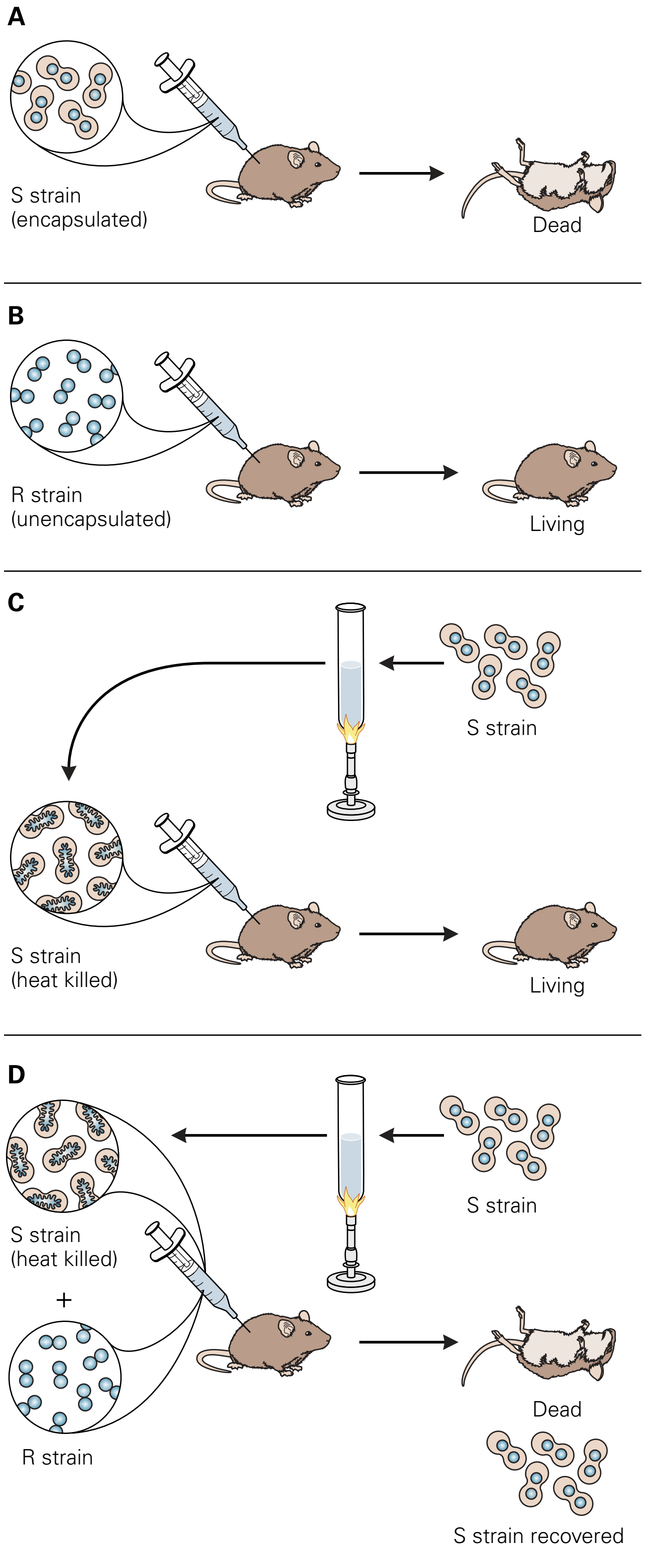
S. pneumoniae, can undergo a form of fratricide, in which the competent cells kill other cells in the population, releasing even more DNA for subsequent uptake by the competent bacteria.

The mechanism of DNA uptake across the inner membrane of most naturally competent Gram-negative bacteria is similar to that of Gram-positive bacteria, and many of the proteins involved in competence are conserved in both systems (Figure 7-9). In both bacteria, the core competence proteins are homologous to the main proteins involved in type 2 secretion systems (T2SS, see chapter 13) and type IV pili (see chapter 11), suggesting that they are evolutionarily related. In Gram-negative bacteria (Figure 7-9A), efficient DNA uptake requires the binding of specific DUSs to the long filamentous type IV pili sticking out of the surface of the cell and the transport of the double-stranded DNA into the periplasm through a secretin (PilQ) pore. Transport is driven by ATP hydrolysis that is coupled to the pili retraction that pulls the DNA into the periplasm, where it interacts with the DNA receptor ComEA. Transport of DNA across the inner membrane of Gram-negative bacteria involves nicking and degradation of one DNA strand by a periplasmic nuclease and funneling of the other strand through the ComEC transmembrane channel. Once inside the cytoplasm, the strand of the DNA gets integrated into the chromosome of the bacterium through homologous recombination. In Gram-positive bacteria (Figure 7-9B), the first step is nonspecific binding of the double-stranded DNA to a type IV-like pseudopilus on the cell surface, which through pilus retraction brings the DNA in contact with the DNA receptor ComEA. The nuclease EndA degrades one strand of the DNA, while the other DNA strand is transported across the cell membrane through the transmembrane channel protein ComEC. Once in the cytoplasm, the single-stranded donor DNA recombines with a homologous region of the chromosomal DNA of the recipient. Recently, another type of natural transformation has been reported for DNA uptake by *H. pylori* and *C. jejuni*, the ComB system, which is closely related to type 4 secretion systems (T4SS) (see chapter 13) and to the VirB/VirD system of *Agrobacterium tumefaciens* for Ti plasmid transfer into plants (see Figure 13-12).

Conjugation: Plasmids and Transposons

Most Gram-negative and Gram-positive bacteria are able to transfer DNA (plasmids and other DNA elements) directly from one cell to another in a process called **conjugation**. It was first suspected in 1947 that bacteria could exchange their DNA when it was observed that mixing two different strains of *E. coli* resulted in new progeny that were unlike either of the parental strains, suggesting some sort of mating process. During conjugation, the two DNA strands separate and one strand moves from the **donor** bacterium into the **recipient** bacterium through a pore in the mating bridge that forms between the two bacteria. The two single strands in each cell then serve as replication templates to generate double-stranded DNA again. The progeny that result from conjugation are called **transconjugants**.

Plasmids are extrachromosomal DNA that can replicate and partition independently of the chromosome. Many



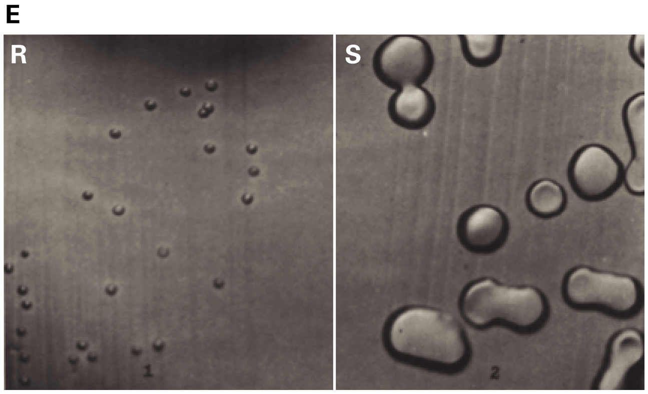


Figure 7-8. Griffith's classic experiment demonstrating natural transformation in Gram-positive *Streptococcus pneumoniae*. (**A**) Mice injected with a pathogenic encapsulated S strain with smooth colony morphology became sick and died. (**B**) Mice injected with a nonpathogenic R strain with rough colony morphology did not die. **(C)** Mice injected with a heat-killed S strain did not die. **(D)** Mice injected with an R strain mixed with a heat-killed S strain got sick and died. Live S strain bacteria were isolated from the dead mice, indicating the conversion of the avirulent bacteria into the virulent, encapsulated form. (Data from Griffith F. 1928. *J Hygiene* **27**:113–159.) **(E)** Left panel: The rough (R) colonies of a mutant serotype 2 strain containing a mutation that blocks capsule formation. Right panel: The same strain as in the left panel but transformed with purified "transforming principle" (i.e., DNA) from another serotype 3 strain, showing restoration of serotype 3 capsule production that results in large smooth colonies. Reproduced from Avery OT, MacLeod CM, McCarty M. 1944. *J Exp Med* **79**:137–158, with permission.

genes required for virulence, including antibiotic resistance genes, some toxin genes, and genes that promote adherence to host cells, reside on plasmids and are frequently acquired by and transferred among commensal and pathogenic bacteria. Often the commensal bacteria in the bacteria-rich gut and other host niches maintain plasmids and thereby serve as "**reservoirs**" for new genetic traits that can spread to other bacteria. Some naturally occurring plasmids are not transmissible (nonconjugative) and do not move to other bacteria, but they are maintained because they encode genes that impart beneficial phenotypes. Other naturally occurring plasmids are either self-transmissible or mobilizable (Figure 7-10). **Self-transmissible plasmids** are a form of **mobile genetic element (MGE)** that encodes genes for a transfer system (called "Tra" system), which is necessary and sufficient for transfer of the plasmid DNA from a bacterial donor cell to a recipient cell by a process broadly described as conjugation.

There are several types of conjugative plasmids that use a related mechanism of DNA transfer from the donor to the recipient cell. The first conjugative plasmids studied in detail were called **F ("fertility")-factors.** F-factors replicate separately from the bacterial chromosome and are transferred by a conjugation mechanism that is briefly outlined

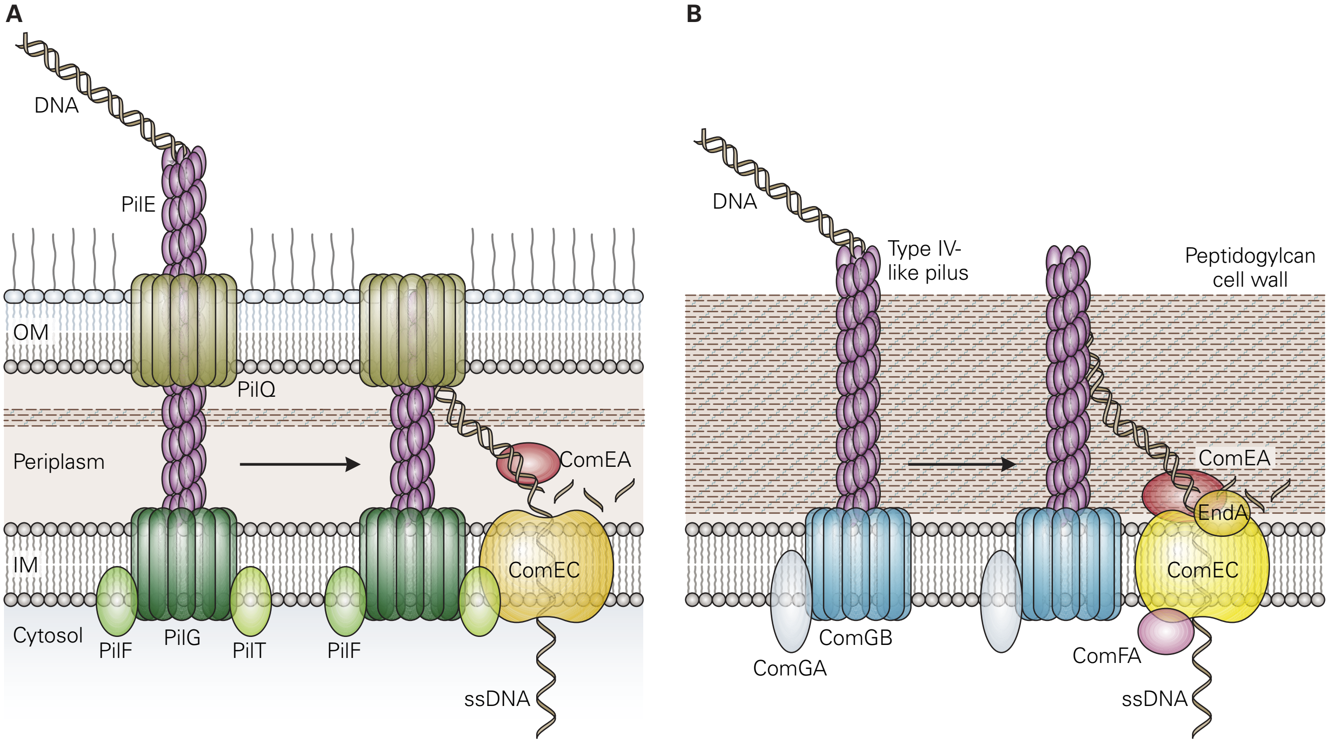


Figure 7-9. General pathways of natural DNA transformation. Shown are the DNA uptake systems for Gram-negative bacteria **(A)** and Gram-positive bacteria **(B)**. Both are comprised of proteins homologous to type 2 secretion systems (T2SS) and type IV pilus biogenesis systems. In each case, pili or pseudopili mediate initial DNA binding, then pili retract and interact with the DNA receptor ComEA complex and the DNA nuclease(s) that nick and degrade one strand of the DNA and facilitate funneling the other strand through the pore formed by the ComEC transmembrane channel. Once in the cytoplasm, the single strand of donor DNA binds to single-stranded binding protein (Ssb), which delivers it to RecA protein for recombination with homologous regions of the recipient chromosomal DNA.

here (Figure 7-10A). Besides genes encoding proteins for plasmid replication, pili that make cell-cell contact, and DNA transfer, F-factors often contain a type of simple transposon called an insertion sequence (see the following discussion). Sometimes, F-factors can also contain complex transposons with genes encoding antibiotic resistances, and transfer of such an F-factor from donor to recipient cells spreads antibiotic resistance.

When an F-factor enters a bacterial cell that has the same insertion sequence or complex transposon in its chromosome, the F-factor can occasionally integrate into the chromosome by RecA-mediated recombination in the transposon sequence. An integrated F-factor is referred as an **episome**, which has two implications to HGT. First, the integrated F-factor can aberrantly recombine out of the bacterial chromosome such that a segment of adjacent bacterial chromosome is now included in the plasmid. This F-factor modified with its bacterial chromosomal genes is referred to as an **F′-factor** (pronounced as “F prime”-factor), which can conjugate into a recipient bacterial cell, thereby transferring chromosomal genes from one bacterium to another. Second, the integrated F-factor episome has no mechanism to sense that it is in a bacterial chromosome. Consequently, DNA transfer can initiate in the incorporated episome and transfer long segments of adjacent bacterial chromosome into the recipient cells. These segments of transferred chromosomal DNA can recombine into the genome of the recipient bacterial cells, thereby horizontally transferring new mutations and genes. Because the frequency of transferring a region of a donor chromosome into a recipient cell by conjugation from an integrated F-factor or episome is much higher than in the case where the F-factor is not integrated into the chromosome, bacterial strains with an integrated F-factor are termed **high-frequency recombination (Hfr)** strains.

Conjugative transposition is another mechanism that transfers plasmids from one bacterium to another. In conjugative transposition, the plasmid, which is referred to as an **integrative conjugative element (ICE)**, is initially integrated at a specific site in the bacterial chromosome. Environmental and stress conditions signal site-specific recombination that excises an ICE from the chromosome into its conjugative plasmid form. As with F-factors, F′-factors, and episomes that initiate Hfr, all the genetic information needed for ICEs to be transferred via conjugation from one bacterium to another is encoded within the ICE plasmid. They all contain an *oriT* site (origin of transfer sequence)

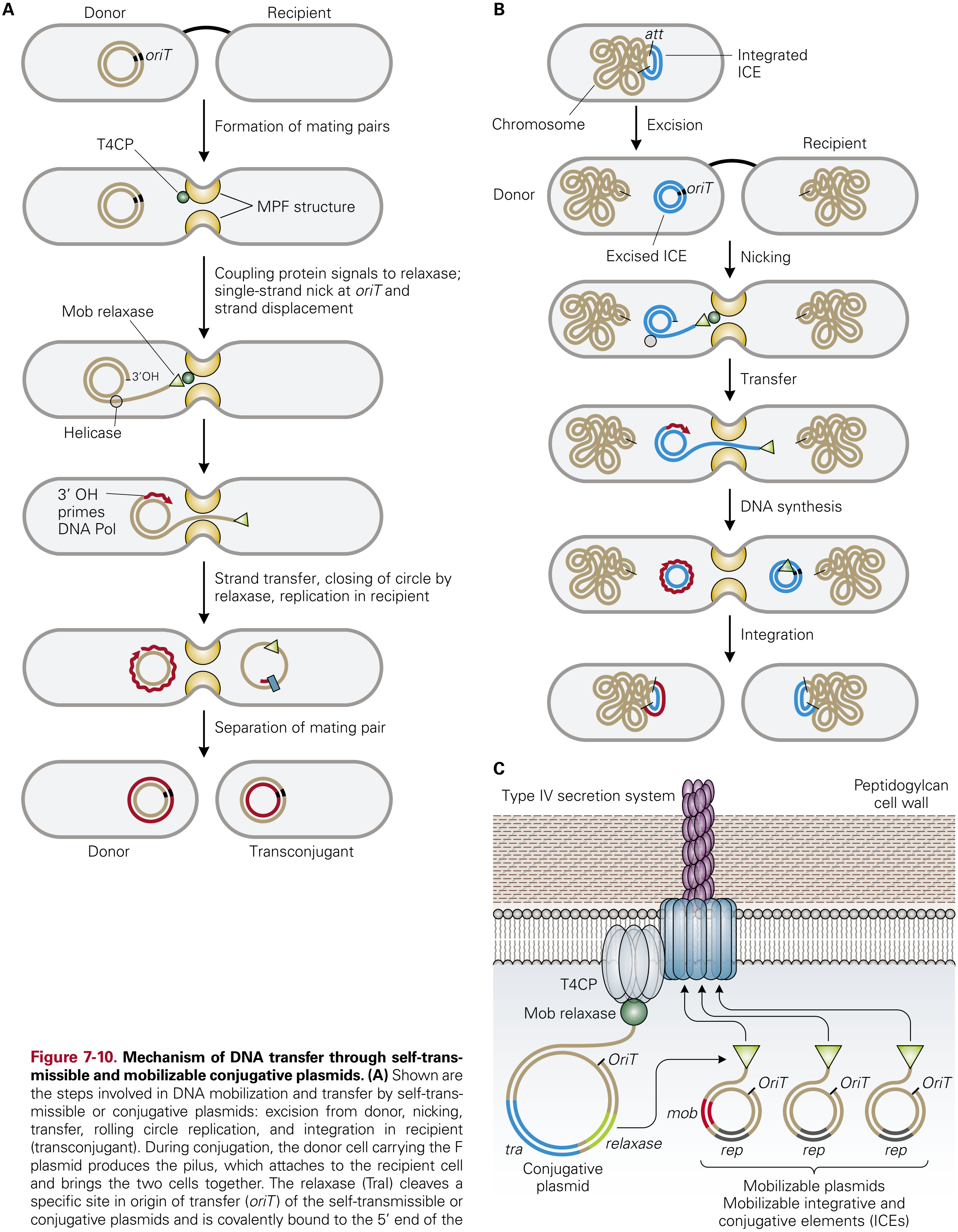

A
Donor
Recipient
oriT
Formation of mating pairs
T4CP
MPF structure
Coupling protein signals to relaxase; single-strand nick at *oriT* and strand displacement
Mob relaxase
3′OH
Helicase
3′ OH primes DNA Pol
Strand transfer, closing of circle by relaxase, replication in recipient
Separation of mating pair
Donor
Transconjugant
B
att
Integrated ICE
Chromosome
Excision
Recipient
Donor
oriT
Excised ICE
Nicking
Transfer
DNA synthesis
Integration
C
Type IV secretion system
Peptidoglycan cell wall
T4CP
Mob relaxase
OriT
OriT
OriT
OriT
mob
tra
relaxase
rep
rep
rep
Conjugative plasmid
Mobilizable plasmids
Mobilizable integrative and conjugative elements (ICEs)

Figure 7-10. Mechanism of DNA transfer through self-transmissible and mobilizable conjugative plasmids. (A) Shown are the steps involved in DNA mobilization and transfer by self-transmissible or conjugative plasmids: excision from donor, nicking, transfer, rolling circle replication, and integration in recipient (transconjugant). During conjugation, the donor cell carrying the F plasmid produces the pilus, which attaches to the recipient cell and brings the two cells together. The relaxase (TraI) cleaves a specific site in origin of transfer (*oriT*) of the self-transmissible or conjugative plasmids and is covalently bound to the 5′ end of the
]

where transfer initiates, and they encode all the genes needed to assemble the machinery to transfer the DNA from the donor bacterium to the recipient, including *tra* genes encoding a conjugative T4SS, a *t4cp* gene encoding an ATPase T4SS coupling protein (T4CP), *raf* genes encoding relaxosome accessory factors (RAFs), and a *mob* gene encoding a relaxase (Mob). The Mob relaxase is an endonuclease that recognizes the *oriT* site and nicks the DNA. RAFs then bind to form a relaxosome complex, which is recruited to the T4SS apparatus via binding to the T4CP, followed by initiation of rolling-circle replication (Figure 7-10B). The resulting single-stranded DNA bound with relaxase at the end is funneled through the T4SS to the recipient cell, where the plasmid is recircularized and replicated. In the recipient cell, site-specific recombination integrates the ICE plasmid into the bacterial chromosome at specific attachment sites, and the integrated ICE replicates with the bacterial chromosome until stimulated to excise. Besides the *tra* genes and genes encoding an integrase and excisionase required for site-specific recombination, ICEs can carry an assortment of additional "cargo" genes encoding antibiotic resistance and virulence factors, which are moved to the chromosomes of recipient bacteria by conjugation and site-specific recombination. In fact, many conjugative plasmids and conjugative transposon ICEs have promiscuous transfer systems that enable them to move DNA between unrelated species as well as between like species. Interspecies transfer of plasmids and transposons plays a major role in the emerging prevalence of antibiotic resistance (see chapter 16).

Finally, **mobilizable** plasmids contain **integrative mobilizable elements (IMEs)** consisting of an *oriT* (origin of transfer) site and a *mob* gene (for nicking at *oriT*), but they lack the *tra* genes that make up the T4SS transfer apparatus. Consequently, mobilizable plasmids can replicate in cells lacking a second, conjugative helper plasmid, but they alone cannot be transferred by conjugation to recipient cells. However, in bacteria containing a second conjugative plasmid, the Tra functions are provided in trans, which allows mobilizable plasmids to be transferred to recipient bacterial cells (Figure 7-10C). Mobilizable plasmids are very useful in biotechnology for transferring cloned genes into recipient bacteria from donor bacteria already containing promiscuous transfer machinery.

Transposons (Tns or "jumping genes"), first discovered in bacteria in the 1970s, are MGEs that can move (transpose) from one place in DNA to another place within the same bacterial cell. That is, transposons can move between sites on bacterial chromosomes or between plasmids and the chromosome and vice versa within the same cell. As noted earlier, transposons can insert into conjugative plasmids and move throughout an entire bacterial population. The smallest type of bacterial transposon, called an **insertion sequence** (**IS**), is an autonomous transposable element that consists of a **transposase** gene, encoding an enzyme that promotes their transposition, and inverted repeat sequences at the flanking ends, which are used to target sequences for insertion in target DNA (Figure 7-11). Upon insertion, the target DNA is duplicated as a direct repeat flanking the integrated IS. Some transposons target fairly specific DNA sequences, whereas others have little or no sequence specificity. It should be noted that when an IS element inserts in the coding region of a gene, the gene is usually inactivated. Therefore, IS elements are not generally tolerated in essential genes. However, the inverted repeats at the end of IS elements can contain promoters that point outward and can drive the expression of adjacent genes in the chromosome or plasmids. Besides IS elements, there are composite (or complex) transposons that consist of two IS elements flanking a central "core" region that contains additional cargo genes, including genes that impart antibiotic resistance or mediate virulence, such as toxin or capsule biosynthetic genes. In the case of composite transposons, both IS elements and the core region move as a unit to a new position in the DNA of a cell. It should be noted that unlike the special case of conjugative transposons (ICEs), which move between bacterial cells, IS elements and composite transposons move within the DNA of a single bacterial cell. Overall, transposons and conjugative transposons are extremely important players in creating genetic and phenotypic diversity and in transmitting antibiotic resistance and virulence genes within and between populations of bacteria.

Figure 7-10. *continued*

resulting single-stranded DNA. The relaxase-bound single-stranded DNA forms a relaxosome (complex of DNA with TraI, TraY, TraM, and the integrated host factor IHF) that interacts with a T4SS coupling protein (T4CP) and a conjugative type 4 secretion system (T4SS) or mating pair formation (MPF) complex. The ATPase activity of T4CP drives the transport of the single-stranded DNA-relaxase conjugate into the recipient cell, where it completes replication to form an F plasmid (transconjugant). Replication of the F plasmid is likewise completed in the donor cell. **(B)** Transposition of integrative and conjugative elements (ICEs). Like conjugative plasmids, ICEs carry an *oriT* site, at which the DNA is nicked and transfer is initiated, and all of the genes needed for conjugation (transfer of DNA from donor cell to recipient cell): Mob relaxase (nuclease that nicks the DNA), relaxosome accessory factor (RAF), T4CP, and T4SS. In addition, ICEs carry genes needed for integration (recombinase/integrase) and excision (excisionase). The attachment (*att*) sites, where excision/integration occur, are indicated. **(C)** Mobilizable plasmids and their corresponding integrative mobilizable elements (IMEs) carry an *oriT* site and a *mob* gene, but they lack *tra* genes and need to use another conjugative transfer system to transfer their DNA to recipients.

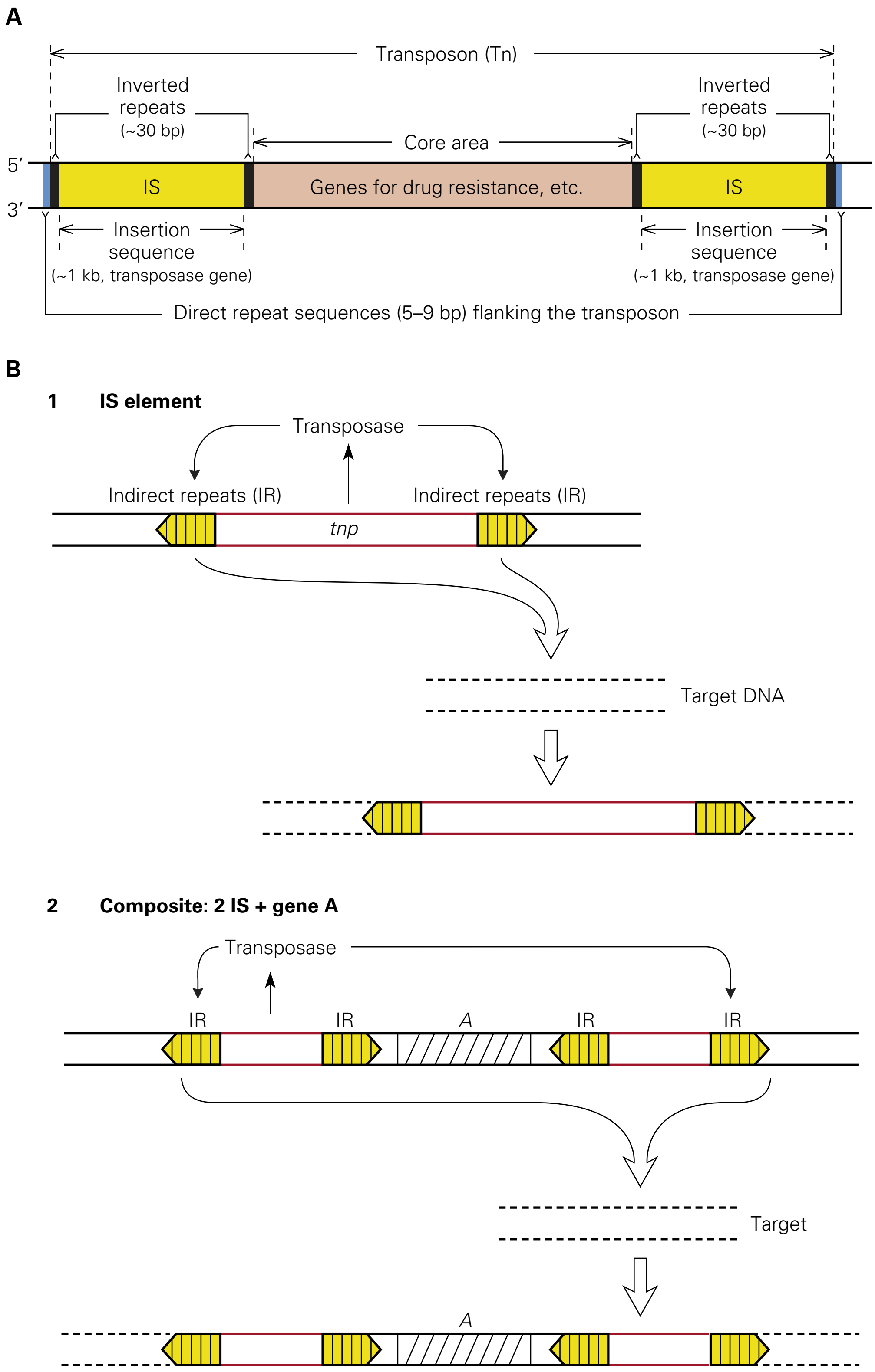

A
Transposon (Tn)
Inverted repeats (~30 bp)
Core area
Inverted repeats (~30 bp)
5′
3′
IS
Genes for drug resistance, etc.
IS
3′
5′
Insertion sequence (~1 kb, transposase gene)
Insertion sequence (~1 kb, transposase gene)
Direct repeat sequences (5–9 bp) flanking the transposon
B
1 IS element
Transposase
Indirect repeats (IR)
Indirect repeats (IR)
tnp
Target DNA
2 Composite: 2 IS + gene A
Transposase
IR
IR
A
IR
IR
Target
A

Figure 7-11. Mechanism of DNA transposition. (**A**) General structure of a transposon. Common features of transposons are: short flanking direct repeats of 3 to 9 bp, a set of inverted repeats of ~30 bp at each end of the ~1 kb insertion sequence that encodes the transposase gene, and a core region that contains additional cargo genes for antibiotic resistance, virulence, and other functions. (**B**) During transposition, a transposon can move from one location to another nonhomologous location on a genome within the bacterial cell. The target DNA sequence is duplicated as a direct repeat flanking the transposon. Many transposons have little or no target sequence specificity. An IS element is the simplest transposon and consists of only the inverted repeats required for transposition and the gene encoding the transposase. IS sequences do not carry a core region. A composite (or complex) transposon consists of two IS elements flanking a core region carrying cargo genes. Composite transposons transpose to new DNA sites as a unit of the two IS elements and the core region.

Phage Transduction

Another mechanism by which bacteria can exchange DNA is through transfer by bacterial viruses (**bacteriophages**, or "phages" for short) in a process called **transduction**. In 1926, it was shown that a filterable agent from scarlet fever isolates could convert non-scarlet fever-inducing strains of *Streptococcus pyogenes* into strains that could induce scarlet fever. This suggested that a bacteriophage-encoded toxin was responsible for the disease. However, it was not until 1951 that the diphtheria toxin *toxA* gene was first demonstrated to be encoded on a β-bacteriophage from *Corynebacterium diphtheriae*. We now know that many toxins and other virulence factors are encoded on integrated bacteriophages found in a wide range of Gram-negative and Gram-positive bacteria.

Transduction allows a bacterium to acquire new DNA segments, usually in the range of 20 to 100 kilobases in length. Some phages are strictly **lytic** phages in that, following infection, they are genetically programmed to replicate their genetic material, synthesize phage structural proteins, and then lyse host bacterial cells to escape. In contrast, some "**temperate**" phages have two phases as part of their life cycle (Figure 7-12). After a temperate phage binds to the bacterial surface receptor and injects the phage DNA into the cytoplasm, depending on the metabolic state of the bacterium, the phage either enters a **lytic phase**, where the phage replicates and lyses the bacterial host, or a **lysogenic phase**, where the phage employs site-specific recombination to integrate into the bacterial chromosome. When lytic phages express their encoded genes, the viral proteins are assembled into capsids and tails (in the case of tailed phages), and the viral genome is packaged into the resulting viral particles (virions). Near the end of the lytic phase, a phage-encoded **holin** inserts into the bacterial cytoplasmic membrane and forms a pore through which a **lysin** is transported to gain access to the bacterial peptidoglycan cell wall. The lysin digests the peptidoglycan, causing rapid cell lysis and the release of the newly synthesized phage into the medium.

Temperate (lysogenic) phages that have integrated into the bacterial chromosome are called **prophages**. A lysogenic phage produces a repressor protein that keeps the phage genome from excising from the bacterial chromosome except under stress conditions that may impair or kill the bacterial host. A lysogenic phage can revert back to the lytic phase, particularly when the bacterium is under stress, but often the integrated phage genome is replicated along with the bacterial chromosome for an indefinite period of time. Once out of the bacterial chromosome, the phage genome directs the lytic phase. Some prophages contain additional genes that, when expressed, confer new biochemical and virulence properties on host bacteria. As prophages stay in the chromosome and replicate with the bacteria over long periods of time, they may mutate and lose the ability to transition to a lytic phase. Such integrated defective prophages become dormant (i.e., inactive and can no longer enter a lytic phase) but can continue to express virulence genes. Over time these virulence gene-encoding prophages, whether defective in reentering the lytic phase or not, are recognized as a form of pathogenicity island (see the discussion below).

Generalized transducing phages accidently transfer a segment of bacterial chromosomal DNA from one bacterium to another during a phage lytic phase. Phage DNA is packaged into capsid phage heads by a "head-full" mechanism based on DNA size. During the lytic phase, a phage-encoded nuclease degrades the bacterial chromosome into pieces of about the same size as the phage genome. Occasionally during the packaging process, a segment of the bacterial chromosome is packaged instead of the phage DNA. These mispackaged phage particles can still inject their DNA into recipient bacteria, but the delivered DNA cannot replicate in the bacteria. Instead, the delivered DNA segments from the donor bacterium can subsequently recombine by RecA-dependent homologous recombination into regions of the recipient's chromosome. Thus, generalized transduction transfers mutations and genes from one bacterium to another. The frequency of phage particles containing mispackaged bacterial DNA is relatively low. However, keep in mind that phages produce a large number of particles during the lytic phase, which increases the chances of this occurring. Generalized transduction was used as a classical genetic method to map the location of mutations in bacterial genomes before the era of recombinant DNA and whole-genome sequencing.

In contrast, **specialized transducing phages** contain an almost complete phage genome plus one or two bacterial genes that were adjacent to the phage attachment site. During lysogeny, the phage genome circularizes and undergoes site-specific recombination into a site with a specific DNA sequence in the bacterial chromosome. During the transition to the lytic phase, most phage genomes excise precisely and are packaged into phage capsid heads. However, sometimes the excision process is imprecise, and one or two genes in a bacterial chromosome adjacent to the phage attachment site are packaged along with the phage genome. These aberrant phages can still infect and lysogenize other bacteria and thereby carry a short region of bacterial DNA from one bacterial cell to another. This form of transduction is "specialized" because only bacterial genes adjacent to the attachment site are accidentally packaged.

Control of Horizontal Gene Transfer

Toxin-Antitoxin Systems—Retaining the Goods

Acquisition of a large extrachromosomal piece of DNA, such as a plasmid, places a substantial metabolic burden on the cell, not just in terms of the energy and machinery needed to replicate and maintain the DNA, but also in terms of the

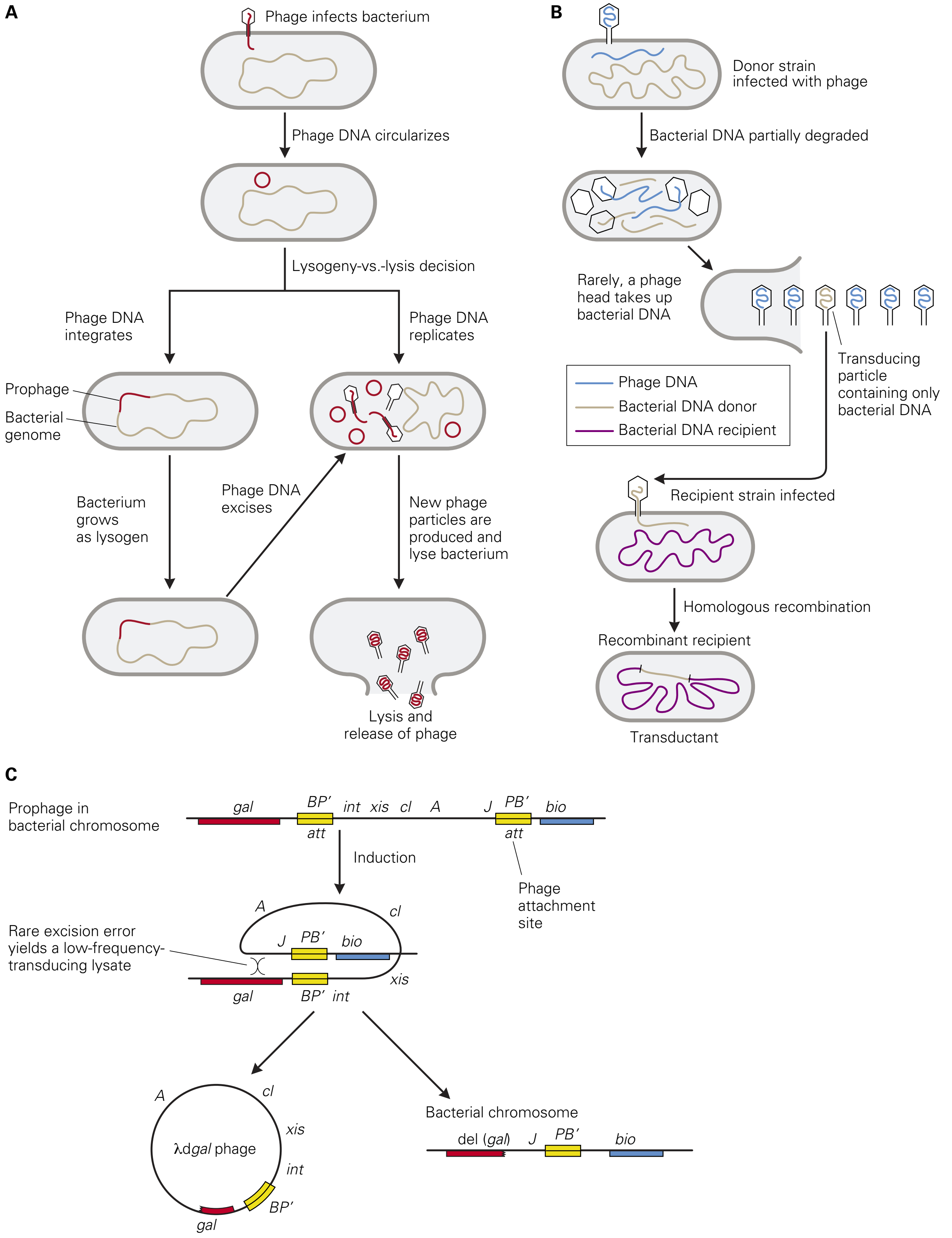

A
Phage infects bacterium
Phage DNA circularizes
Lysogeny-vs.-lysis decision
Phage DNA integrates
Phage DNA replicates
Prophage
Bacterial genome
Bacterium grows as lysogen
Phage DNA excises
New phage particles are produced and lyse bacterium
Lysis and release of phage
B
Donor strain infected with phage
Bacterial DNA partially degraded
Rarely, a phage head takes up bacterial DNA
Transducing particle containing only bacterial DNA
Phage DNA
Bacterial DNA donor
Bacterial DNA recipient
Recipient strain infected
Homologous recombination
Recombinant recipient
Transductant
C
Prophage in bacterial chromosome
gal
BP′
att
int
xis
cI
A
J
PB′
att
bio
Phage attachment site
Induction
A
cI
J
PB′
bio
Rare excision error yields a low-frequency-transducing lysate
gal
BP′
int
xis
A
cI
xis
λdgal phage
int
BP′
gal
Bacterial chromosome
del (gal)
J
PB′
bio

Figure 7-12. Mechanisms of phage transduction-mediated horizontal gene transfer. (**A**) Phage cycle, including lytic phase and lysogenic phase. Bacteriophages can transfer DNA from one bacterial cell to another through two different mechanisms: (**B**) Generalized transduction, where the phage randomly packages some of the bacterial DNA along with its own DNA and (**C**) Specialized transduction, where the phage genes and sometimes genes adjacent to the phage attachment site in the bacterial chromosome are inaccurately excised and packaged into the virion.

cellular effects of the expressed gene products. Even though it might be beneficial for the bacterium to harbor the plasmid because the encoded gene products might confer advantages, such as antibiotic resistance, additional adherence properties, iron or nutrient acquisition systems, or other survival factors, these require a lot of cellular resources to maintain. Without a mechanism for retention, the extra DNA can easily be lost during bacterial replication, placing bacteria without the plasmid at a distinct disadvantage under certain conditions. So, to maintain the plasmid once it has been acquired by a bacterium, many plasmids carry a two-gene operon that encodes a **toxin-antitoxin (TA) system**, where the toxin is a protein or peptide that inhibits an essential cellular function and causes growth arrest or killing of the bacterium, and the antitoxin is a regulatory small RNA (sRNA) that blocks toxin mRNA translation, an RNA that binds to toxin protein, or peptide that binds tightly to the toxin and neutralizes its toxic activity. In a sense, the antitoxin serves as a bacterial "immunity" factor that protects the bacterium from the action of the toxin.

As long as the bacterium retains the plasmid with the TA system during replication, then both toxin and antitoxin continue to be expressed and the antitoxin prevents the toxin from working. However, if the plasmid is lost during replication, then the bacterial progeny cannot make any more toxin or antitoxin. Since the toxin is more stable than the antitoxin, once the toxin-antitoxin dissociates and the antitoxin is degraded, the released toxin will act to block bacterial growth and/or kill the bacterium. Since their first discovery in plasmid maintenance, many different TA systems have been discovered on plasmids, bacteriophages, and chromosomes and have been found to play additional roles in bacterial population survival and persistence under stressful conditions. We will discuss TA systems in more detail in chapter 16.

Restriction-Modification Systems—Bacterial Innate Immunity from Foreign DNA

To prevent acquisition of foreign DNA, most (90%) bacteria and archaea harbor **restriction-modification (RM) systems**, comprised of two cognate genes. One gene encodes a **restriction enzyme**, a nuclease that cleaves double-stranded DNA at a specific recognition site, and another gene that encodes a modification enzyme, a site-specific **DNA methylase** that modifies DNA at the sequence recognized by its cognate restriction enzyme. DNA methylation of double-stranded DNA prevents the restriction nuclease from cleaving the DNA (Figure 7-13). The specific double-stranded DNA sequence recognized by a cognate RM pair is usually 4 to 8 base pairs in length and frequently is found in bacterial or phage genomes. Any invading foreign double-stranded DNA from a phage infection or another source that has not been methylated at the restriction recognition site will get degraded by the restriction enzyme. Consequently, a mutation that inactivates the methylase of an RM pair is lethal and not tolerated.

As expected, phages have evolved a number of ways to evade the RM systems of bacteria, including modifying bases or incorporating unusual bases that protect them from recognition by the restriction nuclease; encoding phage proteins that bind DNA and mask the restriction sites to protect them from restriction nucleases; encoding a phage methylase or stimulating the existing bacterial methylase to also modify the phage DNA; and encoding phage proteins that mimic DNA and neutralize or sequester the RM system nucleases. Importantly, unmethylated foreign DNA that enters recipient cells as single strands through natural transformation or conjugation will be cleaved by restriction enzymes that recognize the double-stranded DNA as their substrate.

CRISPR-Cas Systems—Bacterial Adaptive Immunity from Foreign DNA

As mentioned earlier, although microbes can benefit from HGT, it comes at a cost in terms of fitness, such that sometimes acquiring a genetic mobile element can be detrimental to the bacterium. **CRISPR-Cas systems** (so named for clustered regularly interspaced short palindromic repeats and their associated Cas proteins) are another form of bacterial immunity against acquisition of foreign DNA (Figure 7-14A). CRISPR-Cas modules are found in large multigene operons in most archaea and many bacteria and serve to block invading genetic elements, such as bacteriophages and plasmids. Each CRISPR locus is comprised of DNA direct repeat sequences of 23 to 50 base pairs in length that are separated by similar-sized, nonrepetitive **DNA spacer sequences** that have sequence identity with DNA sequences from plasmids or bacteriophages. These alternating direct repeats (palindromes) and unique spacer sequences (i.e., the CRISPRs) can range from 2 to several hundred within a single CRISPR locus and specify the targets of CRISPR interference, which are foreign DNA to which the bacterium has previously been exposed and is now protected against in the event of invasion. A set of *cas* genes immediately precedes or follows the CRISPR locus and encodes the Cas protein machinery responsible for processing and mediating the CRISPR-mediated interference activity, resulting in degradation of foreign DNA (or in some cases RNA).

There are three main types of CRISPR-Cas systems, defined on the basis of their signature Cas protein with nuclease activity (Cas3, Cas9, or Csm/Cmr, respectively) (Figure 7-14B). The crRNP complexes of type I and type III systems contain multiple Cas subunits involved in assembly of the crRNP and surveillance, whereas the type II system has only one Cas9 protein plus a transactivating crRNA (**tracrRNA**). Extensive comparative genome studies have revealed considerable diversity in CRISPR-Cas systems, so it is important to bear in mind that these three types have many subtypes and variants. New biotechnology applica-

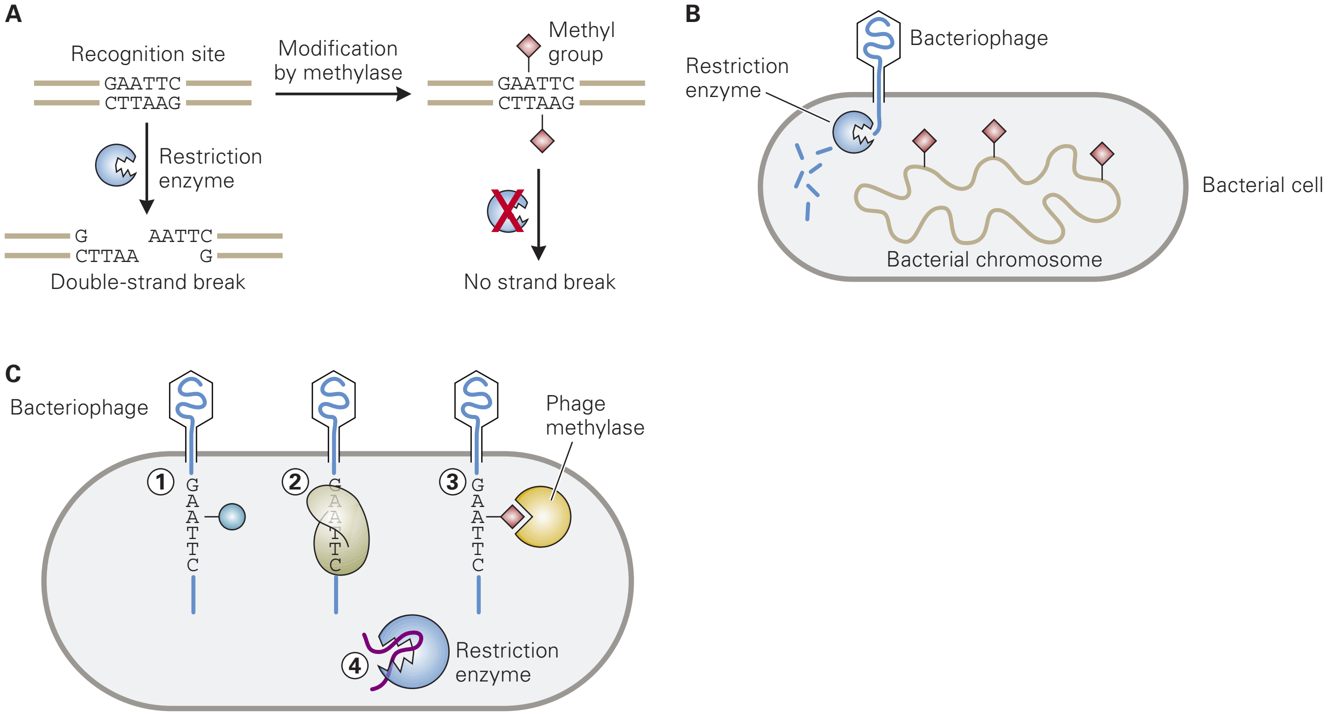


Figure 7-13. Restriction-modification systems. Bacteria harboring genes for restriction-modification (RM) systems are protected from invasion by foreign double-stranded DNA that lack the modification provided by the RM system. **(A)** The RM system is comprised of a site-specific restriction endonuclease (such as EcoRI) that introduces double-stranded breaks at specific EcoRI sites in the DNA and its cognate DNA methylase that modifies the DNA at that same site and protects it from degradation by the nuclease. **(B)** Any foreign double-stranded DNA, such as bacteriophage DNA, that has not been modified by the methylase is subject to degradation by the nuclease upon entering the bacterial cell. **(C)** Strategies used by some phages to evade RM systems include: (1) modifying or incorporating unusual bases (such as a modified A, as shown here) into the DNA, (2) encoding phage proteins that bind to the DNA and mask the restriction sites, (3) encoding phage methylase or stimulating the bacterial methylase to also modify the phage DNA, and (4) encoding phage proteins that mimic DNA and bind to the RM system proteins.

tions of the CRISPR-Cas9 system for genome editing will be discussed in chapter 10.

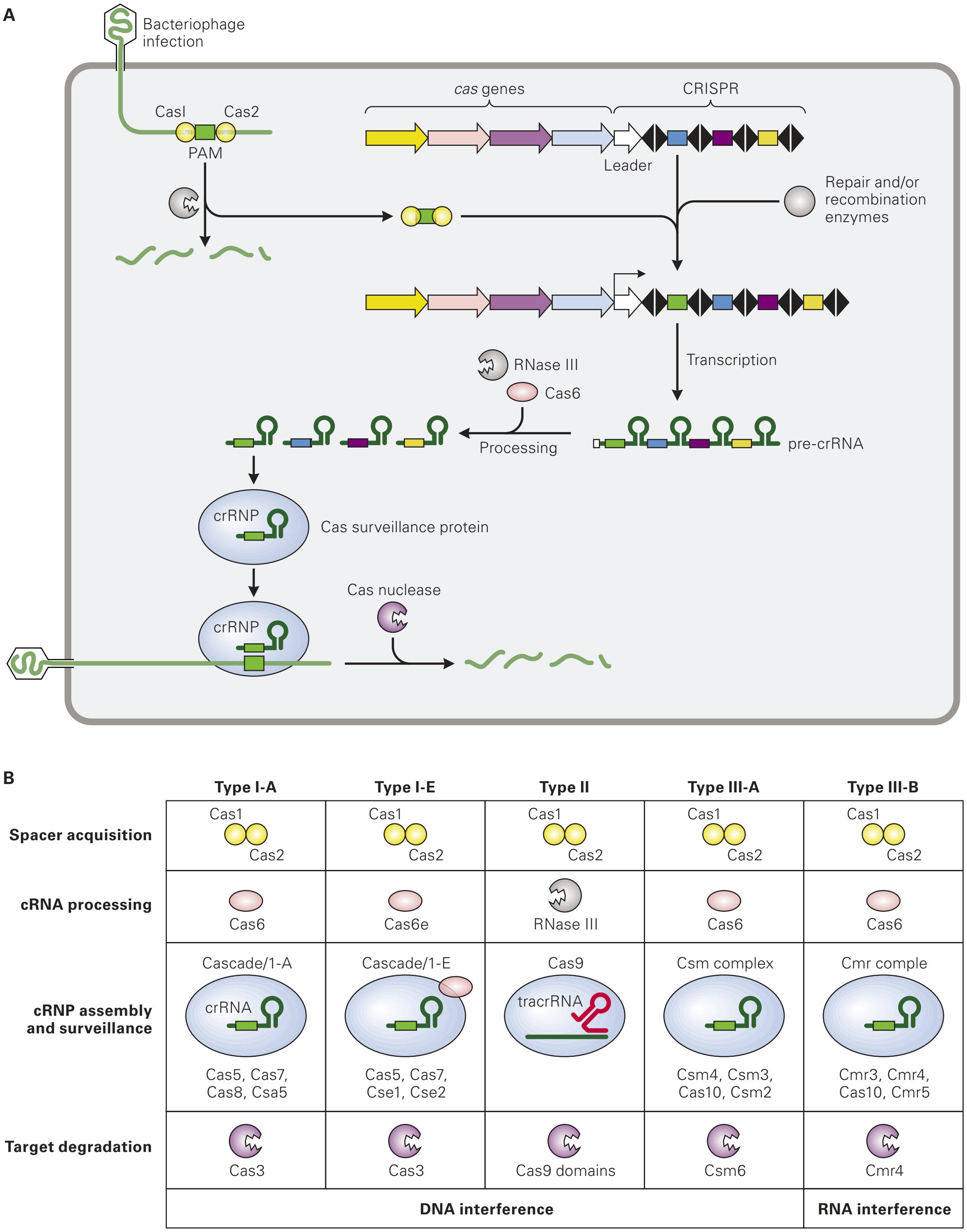
Type 6 Secretion Systems—Bacterial Defense Against Conjugation

The **type 6 secretion system (T6SS)** is a bacterial nanomachine found in a wide variety of Gram-negative bacteria. These bacteria use their T6SS apparatus to antagonize adjacent eukaryotic or bacterial cells by ejecting a toxin-coated "spear" out of the bacterial cell with the T6SS. This spear penetrates the membrane of adjacent cells, delivering to them the toxic proteins that are bound to the spear. In certain *Pseudomonas* species, the T6SS has a special post-translational regulatory system, which senses exogenous membrane perturbations and specifically builds its T6SS machine such that its toxic spears are aimed directly at the source of the membrane perturbation. One type of membrane perturbation recognized by this system turns out to be the mating pair complex formation associated with the **T4SS-mediated DNA conjugation**. Consequently, when a conjugative donor cell attempts to conjugate DNA into these T6SS-positive *Pseudomonas* recipient bacteria, the donor cell is frequently met with a lethal T6SS counterattack, thereby eliminating these donor bacteria from the bacterial population. We will discuss bacterial secretion systems including T6SS and T4SS in detail in chapter 13.

Pathogenicity Islands and Pathogen Evolution

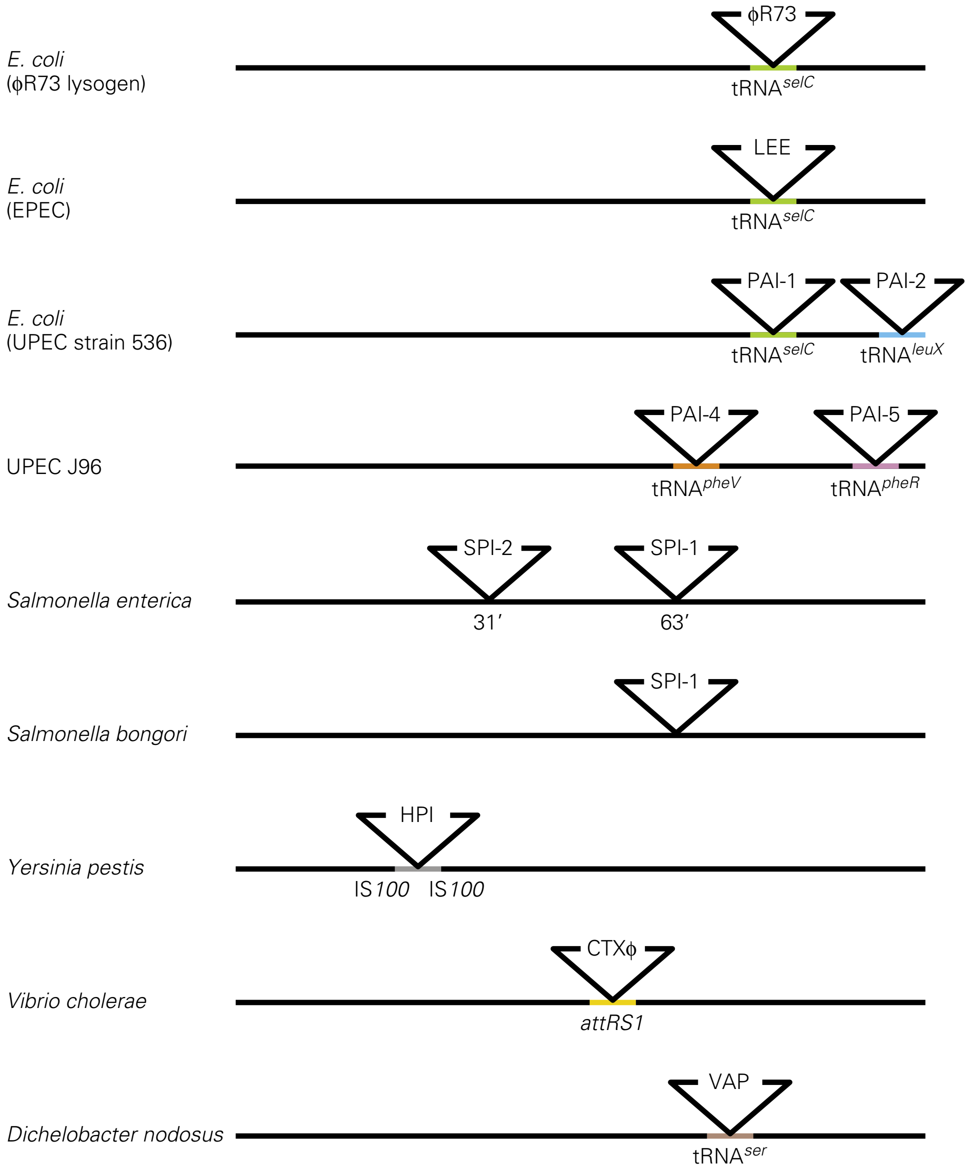
Properties of Pathogenicity Islands

In the early 1980s, studies of *E. coli* strains involved in urinary and intestinal diseases revealed that the *hly* genes, encoding the pore-forming toxin α-hemolysin, were located on large distinct chromosomal DNA regions (**genomic islands**) with a % G+C content and codon usage that was different from that of the rest of the bacterial chromosome. The % G+C content of these DNA regions was found to be 41%, whereas the overall % G+C content of the *E. coli* chromosome is 51%. Further studies showed that additional virulence genes, encoding P fimbriae (adherence proteins), a toxin called cytotoxic necrotizing factor-1, and other virulence-associated proteins, were also located within these regions. These regions were flanked by direct



	Type I-A	Type I-E	Type II	Type III-A	Type III-B
Spacer acquisition	Cas1 Cas2	Cas1 Cas2	Cas1 Cas2	Cas1 Cas2	Cas1 Cas2
cRNA processing	Cas6	Cas6e	RNase III	Cas6	Cas6
cRNP assembly and surveillance	Cascade/1-A crRNA Cas5, Cas7, Cas8, Csa5	Cascade/1-E Cas5, Cas7, Cse1, Cse2	Cas9 tracrRNA	Csm complex Csm4, Csm3, Cas10, Csm2	Cmr comple Cmr3, Cmr4, Cas10, Cmr5
Target degradation	Cas3	Cas3	Cas9 domains	Csm6	Cmr4
	DNA interference				**RNA interference**

Figure 7-14. CRISPR-Cas system-mediated adaptive immunity in bacteria. (A) CRISPR-Cas systems are comprised of CRISPR RNAs (crRNAs) encoded by the CRISPR locus of alternating direct repeats and unique spacer sequences, flanked upstream (or downstream) by *cas* genes that encode CRISPR-associated (Cas) proteins. Spacer acquisition occurs through recognition of a protospacer adjacent motif (PAM) in the invading DNA (green) by Cas1 and Cas2 spacer acquisition proteins (yellow) that target the DNA for digestion and assemble the unique spacer sequences into the CRISPR locus after the Leader sequence, with the help of bacterial repair and/or recombination enzymes (gray). Transcription of the CRISPR locus yields a single pre-crRNA transcript that is then processed by Cas6 crRNA-processing proteins (pink) or bacterial housekeeping RNase III (depending on the type of CRISPR-Cas system). Mature crRNAs and Cas surveillance proteins (blue) form CRISPR ribonucleoprotein (crRNP) complexes that scan invading DNA for complementary DNA sequences, which are then targeted for degradation by Cas nucleases (purple). **(B)** Three types of CRISPR-Cas systems, Type I (I-A or I-E shown here), Type II, or Type III (III-A or III-B shown here), are defined based on their characteristic Cas nuclease protein (Cas3, Cas9, or Csm6/Cmr4, respectively). Type I and Type III systems have multiple Cas subunits, whereas Type II has a single Cas9 protein. Type III-B is unique in that it targets RNA, rather than DNA, for degradation. Adapted from van der Oost J, Westra ER, Jackson RN, Wiedenheft B. 2014. *Nat Rev Microbiol* **12:**479–492, with permission.



E. coli (ϕR73 lysogen)
ϕR73
tRNA^selC
E. coli (EPEC)
LEE
tRNA^selC
E. coli (UPEC strain 536)
PAI-1
PAI-2
tRNA^selC
tRNA^leuX
UPEC J96
PAI-4
PAI-5
tRNA^pheV
tRNA^pheR
Salmonella enterica
SPI-2
SPI-1
31′
63′
Salmonella bongori
SPI-1
Yersinia pestis
HPI
IS100
IS100
Vibrio cholerae
CTXϕ
attRS1
Dichelobacter nodosus
VAP
tRNA^ser

Figure 7-15. Pathogenicity islands. Pathogenicity islands (PAIs) can encode many virulence genes in compact, distinct genetic units that are inserted into defined locations on the chromosome, flanked by tRNA genes (*leuX, selC*), phage/plasmid attachment sites (*att*), or plasmid/transposon insertion sequences (IS). Stepwise acquisition of PAIs can lead to progressive increase in virulence. Adapted from Groisman EA, Ochman H. 1996. Pathogenicity islands: Bacterial evolution in quantum leaps. *Cell* **87:**791–794, with permission.

repeats of 16 to 18 base pairs, indicating that these DNA sequences had been acquired through HGT (i.e., recombination, phage insertion, or transposition). In the early 1990s, these DNA segments of the genome containing one or more virulence genes acquired through HGT were named **pathogenicity islands (PAIs)**.

Comparing a large number of genome sequences, including multiple sequences from multiple strains of the same bacterial species, has revealed the importance of PAIs in the diversification of strains within a bacterial species. We now know that the genomes of most bacterial pathogens contain multiple PAIs, whereas their nonpathogenic counterparts do not. Many PAIs appear by sequence analysis to be prophages or remnants thereof, but some appear to be integrated conjugative elements. PAIs can constitute as much as 20% of a bacterial genome. PAIs have been found in both Gram-positive and Gram-negative bacteria and are usually inserted into defined locations within highly conserved DNA regions on the chromosome, such as phage attachment (*att*) sites, bacterial tRNA genes (*leuX, selC*), or insertion sequences (direct repeats) (Figure 7-15). Nevertheless, many PAIs are still part of active mobile DNA elements (plasmids, ICEs, transposons, or phages) or of mobilizable DNA elements and have a tendency to excise from the chromosome at frequencies as high as 10^{-4} to 10^{-5} or to undergo duplications and amplifications.

PAIs that are still mobilizable (plasmids, transposons, or prophages) carry genes needed for transmission, although many have lost them or have defective ones. Of more interest in terms of virulence, many PAIs encode genes that confer a variety of new functions. These genes may provide the bacteria with a selective advantage under certain environmental conditions. For example, they may provide new types of pili for altered adhesion, allowing the bacteria to bind to different tissues and enhance colonization. They may provide means for acquiring iron and other nutrients. They may also allow for increased survival in the host by encoding genes for novel surface structures such as modified LPS for increased serum resistance, for capsular biosynthesis to prevent phagocytosis, or for delivery of proteins that enhance bacterial invasion, modulate intracellular signaling processes, or dampen immune responses (e.g., type-3 secretion systems and their secreted proteins—more on these later).

Genes encoding most bacterial protein toxins are located on PAIs. Indeed, lysogenic prophages carrying toxin genes are thought to serve as a major natural reservoir for toxin genes in bacterial populations. HGT of toxin genes in natural environments may account for the prevalence of related toxins among diverse pathogens (see Table 12-1 in chapter 12). For example, the cholera toxin gene (*ctx*), which is phage encoded in *Vibrio cholerae*, is closely related to the heat-labile enterotoxin genes (*elt* or *etx*) found in different strains of *E. coli* that cause diarrhea. These enterotoxin genes can be found either encoded on a plasmid or on the chromosome, depending on the strain of *E. coli*. Other examples are the widespread occurrence of Shiga toxin-related (*stx*) genes in *Shigella* and *E. coli* strains and the closely related botulinum or tetanus neurotoxin (*bot* or *tet*) genes found in different strains and species of *Clostridium*. Considerable evidence suggests that phages containing *stx* from *Shigella* and *E. coli* can be transmitted not only between different bacteria in the intestine of humans and other animals, but also in external aquatic environments, such as sewage, water, and soil contaminated with feces. The diverse locations of the PAIs encoding *Clostridium botulinum* and *Clostridium tetani* neurotoxin genes, which in different strains are found on plasmids or lysogenic prophages or as gene clusters on the chromosome (as putative transposons or prophages), illustrate the degree of HGT that has contributed to their evolution.

Pathogen Evolution in Quantum Leaps

Point mutations, genomic rearrangements, and antigenic variation lead to slow adaptive evolutionary changes, but acquisition of a single PAI can convert a nonpathogenic bacterium into a pathogen in a single step. One of the first demonstrations of this was that of the phage-encoded diphtheria toxin, which is the primary virulence factor responsible for disease symptoms. Nontoxigenic strains of *C. diphtheriae* are avirulent and indeed are often found colonizing the upper respiratory tract. Conversion into virulent toxigenic strains can occur by acquisition of the toxin gene via phage transduction. Plasmids, phages, and transposons are thus means for rapid evolutionary change. These mobile elements, sometimes resulting in PAIs, can contribute in a single step to the ability of the newly formed strains to enhance colonization, survival, and dissemination in the host. Sometimes more than one type of trait is acquired in a single step. Thus, mobile elements have high potential for generating new pathogens in relatively short periods of time. Since gene clusters in PAIs are acquired as a unit in a single HGT integration event, virulence genes carried on PAIs found in different bacterial genera have remarkable sequence similarity and are often found arranged in the same order (i.e., they share a common genetic organization and evolutionary origin).

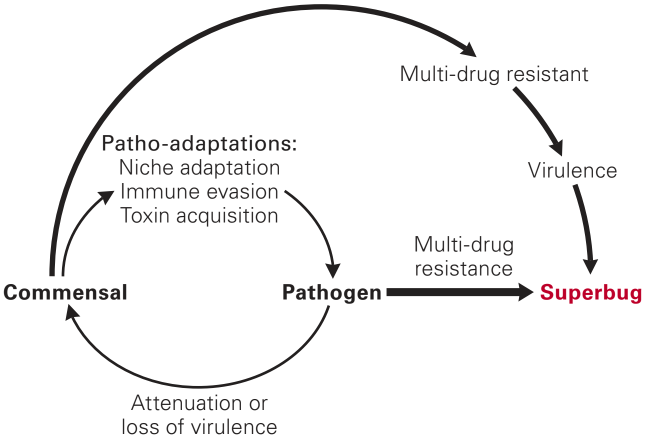


Figure 7-16. Pathoadaptation model for bacterial pathogen evolution. Genetic plasticity and bacterial metagenomes allow bacteria to evolve, sometimes rapidly, in order to survive under the new environmental and stress conditions. This process can turn a benign commensal bacterium into a life-threatening pathogen with new virulence properties, such as adhesins, toxins, and/or other immune-evading factors, or with new survival properties, such as antibiotic resistance, biofilm formation, invasion, and/or persistence. Sometimes when a pathogen enters a new niche and establishes itself, some of the virulence traits it might possess can be lost, resulting in adaptation and attenuation into a commensal. However, when multiple virulence and survival properties are combined, the pathogen can convert into a so-called superbug that is extremely difficult to control.

Stepwise acquisition of PAIs can lead to progressive increase in virulence and the rapid emergence of new pathogens (see Figures 7-15 and 7-16). For example, two large PAIs have been identified in *Salmonella*, each contributing to a specific step in the course of infection. The *Salmonella* pathogenicity island-1 (SPI-1) encodes about 25 genes, including a type-3 protein secretion system and various effector proteins delivered into the mammalian host cells via the SPI-1 type-3 secretion system (see chapter 13). SPI-1 confers the ability to invade epithelial cells. The *Salmonella* pathogenicity island-2 (SPI-2) encodes about 15 genes, including a second type-3 protein secretion system, a two-component regulatory system, and other effector proteins. SPI-2 confers the ability to survive within macrophages and cause systemic infections. Acquisition of these two PAIs was critical in the development of *Salmonella* as an intracellular pathogen because these PAIs enable *Salmonella* to invade host cells, evade host defenses, and cause systemic infections. SPI-1 is present in strains from all subgroups of *S. enterica*, but SPI-2 is not found in *Salmonella bongori* strains, which are of intermediate virulence and are thought to represent an intermediate step in *Salmonella* evolution.

A major take-home lesson of HGT and the multiple mechanisms of genetic exchange in bacteria is that there is tremendous diversity within bacterial species, as well as the more traditional view of differences between bacterial species. Two different clinical isolates that are the same bacterial species based on 16S rRNA gene sequence analysis can show considerable differences in the numbers and types of PAIs, lysogenic bacteriophages, and extrachromosomal elements they contain. These differences underlie the fact that single bacterial species cause a multitude of infections. For example, there are multiple diseases caused by different kinds of *E. coli*, *S. aureus*, *S. pneumoniae*, and *S. pyogenes*, to name a few. This diversity within bacterial species and mechanisms of exchange of large chromosomal segments, such as occurs with natural transformation, bacteriophage-mediated transduction, and conjugation, mean that bacterial pathogens actually have a much larger gene pool, called a **supragenome or pan-genome**, than what is in the chromosome of any single bacterial cell. The supragenome within a bacterial species and the potential to exchange genes between different bacterial species (often called the **metagenome**) discussed in this chapter present a formidable challenge. Bacterial diseases will continue to emerge, re-emerge, and change due to tremendous genetic plasticity of supragenomes and the vast number of genes available in the metagenome. Indeed, HGT, genome plasticity, and the existing diversity of the available gene pool make bacterial adaptation inevitable in the face of changing environmental and stress conditions and are a major driving force of pathogen evolution and the emergence of the so-called superbugs (Figure 7-16), which we will learn more about in later chapters.

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Questions

1. Should microbiologists be alarmed about the unknown risks posed by bacteria gaining potential virulence traits from external environmental sources?
2. Is acquisition of a PAI sufficient to transform an organism into a pathogen?
3. What is the advantage of incorporating virulence genes into the chromosome over retaining them on plasmids?
4. You have identified an operon on a new strain of *E. coli* that encodes for three genes involved in capsule biosynthesis as well as a regulatory gene. After careful analysis of the operon and its flanking DNA sequences, you suspect that the operon is located on a pathogenicity island that is a lysogenic prophage. What possible evidence might have led you to propose this?
5. *Neisseria* species are known to undergo both phase and antigenic variation. Define phase versus antigenic variation and describe how each might occur. What advantage do they provide for a pathogen?
6. What role have bacteriophages played in pathogen evolution?
7. Some researchers have tried to identify the pathogenicity islands in *Salmonella* by comparing the *E. coli* K12 genome with that of *S. typhimurium*. What are the advantages and disadvantages of this approach?
8. Which types of variation in surface antigens would still be seen in a strain of *N. gonorrhoeae* that was deficient in the ability to carry out homologous recombination?
9. Bacterial cells have the ability to change the set of genes that they express in response to changes in the environment. Explain why the ability to turn on only those genes that are needed at a given time is an advantage to the bacterium.
10. Joe is a sexually promiscuous 22-year-old, who has had gonorrhea five times in the past two years. Each time he went to the doctor, he was given antibiotics and the infection cleared up. List at least two reasons why *N. gonorrhoeae* was able to cause recurrent infections in Joe. Could Joe be vaccinated to avoid these recurring infections? Why or why not?
11. Secretion of DNase into the extracellular medium by *S. aureus* would most likely be able to prevent transfer of DNA by
    a. conjugal transfer by a self-transmissible plasmid.
    b. conjugal transfer by a mobilizable plasmid.
    c. natural transformation.
    d. all of the above.
    e. none of the above.
12. Which type of recombination does not require RecA or long stretches of homologous sequences and is important for the integration of bacteriophage genomes into bacterial chromosomes or plasmids?
    a. Mutagenic recombination
    b. Homologous recombination
    c. Replicative recombination
    d. Generalized recombination
    e. Site-directed recombination
13. Which of the following statements is/are correct with regard to CRISPR-Cas systems?
    a. CRISPR-Cas systems protect bacteria and archaea from viral infection.
    b. CRISPRs code for Cas proteins that can help destroy invading viral DNA and bacterial mRNA.
    c. CRISPRs can be used to infer viral infection history.
    d. None of the above are correct.
    e. A and B are correct.
    f. A and C are correct.
    g. B and C are correct.
    h. A, B, and C are correct.

Solving Problems in Bacterial Pathogenesis

1. Autolysis and natural competence contribute to *Neisseria's* ability to undergo antigenic variation, an important virulence factor that allows it to avoid an effective immune response by the host and also contributes to cell and tissue tropism. To determine the importance of RecA-dependent recombination in antigenic variation in *Neisseria*, you make a mutant of *Neisseria* lacking the *recA* gene, and you develop a PCR method for detecting whether homologous recombination has occurred. The method you develop involves using a primer to the N-terminal region of the *pilE* gene and a set of primers to the C-terminal regions of several of the *pilS* genes. You find that the PCR reaction using these primers yields a product for the wild type *Neisseria* strain but not for the *recA*$^-$ strain. You conclude that RecA-dependent recombination is important for antigenic variation to occur. Explain the basis of the experimental design that allowed you to come to this conclusion (i.e., how could you tell whether antigenic variation occurred using this PCR method?).

2. In the early days of the study of genetic variation in *Neisseria*, there was a debate about whether most of it was due to intragenic or intergenic recombination. Design a simple experiment to distinguish between these two possibilities. How would the outcome look if *both* were making comparable contributions?

3. You do a transposon mutagenesis experiment on bacterium X. You are interested in mutants that have decreased ability to produce a toxin. In several cases, you find that toxin production has disappeared. In one case, however, the toxin is produced, but at a lower level than the wild type strain. Also, whereas toxin production in the wild-type strain was triggered by the exposure of the bacteria to low iron conditions, the mutant makes as much toxin in high iron medium as in low iron medium. How could you explain these two types of mutants? Why might the second type of mutation (constitutive expression of the toxin gene) reduce the ability of the bacterium to cause disease?

4. The production of hemagglutinins, which are bacterial adhesins for mammalian cells, is a well-established virulence factor for a number of bacterial pathogens. Thus, it was not surprising that *Porphyromonas gingivalis,* a Gram-negative anaerobic bacterium associated with dental plaque and periodontal disease, also expresses several hemagglutinins on its cell surface. It was found that one of the hemagglutinin genes (*hagA*) of *P. gingivalis* contains four large contiguous direct repeats varying from 1,318 to 1,368 bp in length, which together encode a 2,628-amino acid protein of 283.3 kDa (a very large protein!). The repeat unit (denoted as *HArep*), which contains the hemagglutinin adhesin domain, was also found to be present in several other protease and hemagglutinin genes in *P. gingivalis*. The beginning amino acid sequence encoded by the first repeat (*HArep1*) is PNPNPGTTT, while that of the other three repeats (*HArep2-4*) is GTPNPNPNPNPGTTT. The amino acid sequence at the C-terminus of the fourth repeat (*HArep4*) is GTPNPNPNP. Provide a possible mechanism that could account for the presence of this repeat unit four times in *hagA* and also in the other protease and hemagglutinin genes (from a molecular evolutionary point of view).

5. Brucellosis caused by Gram-negative *Brucella* species is a zoonotic disease with serious impact on the livestock industry. In animals, brucellosis can lead to abortions in females and sterility in males. In humans, brucellosis is rarely fatal, but it does cause systemic, febrile (fever-causing) disease that can be debilitating. *Brucella* enter macrophages, but are able to evade phagolysosomal fusion and so are able to survive intracellularly. *Brucella*, unlike other Gram-negative bacteria such as *E. coli*, have an unusual LPS that does not activate the innate immune responses. Comparison of the sequenced genomes of several strains that are nonpathogenic to humans (*B. neotomae* and *B. ovis*) to those that are pathogenic to humans (*B. melitensis*, *B. abortus,* and *B. suis*) has revealed that *Brucella* species are highly conserved overall (> 90% of the genes share 98% to 100% sequence identity at the nucleotide level, with G+C% = 57.3), except for certain distinct regions, called genomic islands (GIs). A group of researchers hypothesized that host specificity and virulence differences must stem from the limited genome diversity found in 5 of these regions that are present in all pathogenic strains: GI-1 (8.1 kb, 9 ORFs, G+C% = 53.2), GI-2 (15.1 kb, 20 ORFs, G+C% = 51.3), GI-3 (21 kb, 30 ORFs, G+C% = 52.3), GI-5 (44.1 kb, 42 ORFs, G+C% = 57.2), and GI-6 (7.5 kb, 10 ORFs, G+C% = 54.2). Comparing these GIs among the species to that of the human pathogen *B. melitensis*, the researchers found that GI-6 is absent in *B. neotomae* and GI-1, GI-2, and GI-5 are absent in *B. ovis*. A schematic diagram of the genetic organization of the ORFs within GI-2 is presented in Figure 1. Based on sequence homology to other genes in the DNA database, the researchers were able to annotate some of the ORFs present. The arrows indicate the direction of transcription.

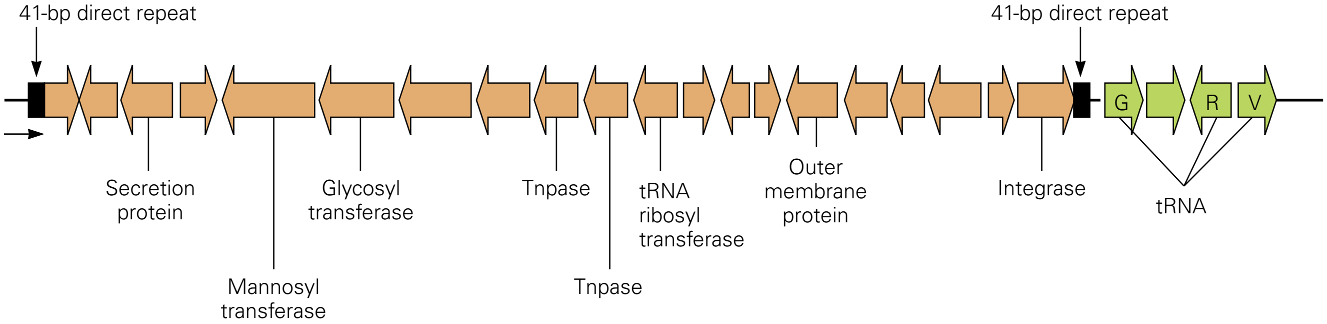

Figure 1. **Organization of ORFs within GI-2.**

a. Considering all of this information, name at least 5 features of GI-2 that led the researchers to propose that GI-2 might be a PAI. What is the most likely mode of horizontal gene transfer for GI-2 if it is a PAI? Be sure to provide your rationale.
b. The researchers suspect that GI-2 may contain genes that contribute to the unusual LPS found in *Brucella* species. What led them to suspect this? (Hint: How does *E. coli* LPS stimulate innate immunity?)
c. Describe an experiment that the researchers could perform to demonstrate that GI-2 indeed contains genes responsible for making the unusual LPS and that this unusual LPS is important for dampening the innate immune responses and enhancing survival in macrophages.
d. Describe an experiment that the researchers could perform to demonstrate that only GI-2 and not any of the other GIs (GI-1, GI-3, GI-5, or GI-6) are involved in pathogenesis.

8

IN THIS CHAPTER

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- Gentamicin Protection Assay for Cell Adhesion and Invasion
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CHAPTER 8

Identification of Virulence Factors

MEASURING INFECTIVITY AND VIRULENCE

Are you a human or a mouse? So goes the traditional challenge to stand up and fight. The answer is supposed to be "a human, who will be big and strong." But today, that question has a different answer. The new answer is: Well yes, I am a human, but sometimes I am a mouse. But also, sometimes I am a fish or a fly or even a worm. This answer is not meant to impugn the integrity of the person asked, but rather to point out a different issue. In standing up for humanity, it is necessary to understand an old and persistent threat, worse than any human terrorist—infectious disease. In the battle against pathogens, sometimes nonhuman experimental animals must take the place of humans, and then a scientist must ask whether a mouse, a fish, a fly, or a worm is the same as a human.

How Does One Experimentally Measure Virulence and Satisfy Koch's Postulates?

Koch's third postulate states that an organism isolated from a diseased individual should cause the same disease when inoculated into a new susceptible host. However, this is only the beginning of the story. Once a disease agent is identified, it is important to determine how this agent causes the disease. This process usually involves recapitulating the disease in some sort of experimental animal model to measure the extent and severity of the disease, identifying factors that contribute to this virulence (**virulence factors**), defining steps in the disease process and progression, and finding prevention and treatment strategies. As the number of methods for probing mechanisms of virulence has expanded, so too has the variety of experimental animal models. In addition to well-recognized laboratory rodent animals, animals ranging from single-cell amoebas to invertebrate insects and worms to vertebrate fish and primates are all commonly used by scientists to investigate various disease mechanisms.

Because virulence is a microbial attribute that is exhibited only in the context of a susceptible host, it is critical that when designing experiments, a suitable infection model system be used for measuring virulence. First, the animal host must be susceptible to the pathogen. Ideally, infection using this animal model should mirror all of the disease symptoms that occur during natural infection in a human. Alas, this is not always possible, and alternative infection models must then be found. Moreover, to identify virulence factors and understand their roles in the infection process that lead to disease, one must also have some way to accurately and reproducibly measure virulence using the infection model. In this chapter, we describe various animal models (and alternatives) that have been developed and are being used for studying infectious diseases caused by bacteria. We also describe various methods that can be used to measure virulence and infectivity (i.e., to determine the degree to which a host has been infected) and identify potential virulence factors.

Animal Models of Infection

Human Volunteers

We start with the best possible model for studying human disease: humans. Human volunteers can be, and have been, used in many infectious disease studies. Ethical considerations make this approach problematic, but it is possible when the disease is not life-threatening, is easily treatable, or can be prevented with a vaccine. Indeed, human volunteers have played an important role in testing of treatments and vaccines against cholera, chancroid (genital ulcers), and gonorrhea, all diseases for which there is no suitable animal model. Additionally, antibiotic intervention trials to assess the efficacy of antibiotic treatments for ulcers or atherosclerosis are also ethically defensible, because the treatment presumably does no harm to the participants and may even benefit them.

Some human trials, on the other hand, have raised serious ethical issues. An example is provided by the large trials in Thailand and Africa in the 1990s of a short course of AZT therapy to prevent mother-to-infant transmission of HIV. Developing countries at that time could not afford to use the long-term AZT regimen used in developed countries to prevent maternal transmission of HIV, a regimen that starts early in pregnancy and continues for months after childbirth. Physicians had begun to suspect that a much shorter course of AZT would protect newborns nearly as well and a lot more cheaply. The controversy arose because the control group received a placebo, whereas the trial group received the short-term course of AZT. Although this seems at first glance to be a rational trial study design, it would not be approved in any developed country. An ethically acceptable test design in developed countries would be to compare women treated with the long course of AZT, a treatment known to be effective, with those treated with the shorter course of AZT. Scientists in the United States and Europe questioned the ethics of the short course trial on the basis that the trial applied a different ethical standard to people in developing countries than the one applied to people in developed countries.

Why were scientists in developed countries, especially the United States, so concerned over the ethical issues raised by this test? One reason stems from an unethical human trial called the Tuskegee syphilis experiment, which was conducted in the United States under the auspices of the precursor of today's National Institutes of Health (NIH). In this trial, men with syphilis were not treated with antibiotics, but instead were merely observed as the disease took its long-term course (see Box 8-1). This "experiment" was initiated in the 1930s, but continued until the first accounts of it appeared in the news in 1972. Two other unethical experiments took place in the 1960s. One was the Jewish Chronic Disease Hospital study, in which chronically ill patients were injected with live cancer cells to see if the cancer cells would be rejected. In the second study, mentally handicapped children newly admitted to the Willowbrook State School in New York were injected with viable hepatitis virus to study the course of infection.

The examples described above are only a few of the historical events that eventually led to the current ethical guidelines and federal regulations concerning human subjects research, which were summarized in the **Belmont Report** of 1979. This report, written by a National Commission for the Department of Health and Human Services, identified three ethical principles that must be maintained during the conduct of research involving human subjects: respect for persons (informed consent), beneficence (maximize benefit, minimize harm), and justice (fair treatment and risk distribution among potential participants). Although most scientists practicing today were not around when these infamous examples of unethical science were perpetrated, the chilling implications of them are still very real to older scientists, who are determined that nothing like them will ever be repeated. Thus, today there are very strict guidelines for conducting human subjects research. In the United States, the **Common Rule** was established in 1981 as a baseline standard of ethics governing biomedical and behavioral research involving human subjects that, in addition to the three ethical principles, includes additional protections for certain vulnerable populations. It also dictates that all government-funded human subjects research must be approved in advance by institutional committees, called **Institutional Review Boards (IRBs)**, made up of experts in performing and interpreting these types of experiments, including physicians, statisticians, and bioethicists. IRBs also enforce rigorous regulations and criteria established by the NIH and other funding agencies for the design, implementation, and reporting of experiments involving human subjects.

A clinical trial study involving the inoculation of humans with a disease agent or the treatment of already infected humans with a new drug or vaccine is called a "**prospective**" **study,** because it starts in the present and moves into

Box 8-1.

The Tuskegee "Experiment": A Shameful Chapter in Infectious Disease History

Syphilis, a sexually transmitted bacterial disease, was widespread in the southeastern United States in the 1930s. The first treatment for syphilis, arsphenamine (an arsenic compound) and bismuth, was introduced in the early 1930s. In 1932, a decision was made by the U.S. Public Health Service in Alabama to withhold treatment from 399 African-American men with syphilis for the purpose of learning more about the development and pathology of the disease. The men, most of whom were poor sharecroppers and could not afford medical care, were offered "free health care," something unknown in their experience. This care consisted of regular visits to a clinic, where they were treated for any ailments (other than syphilis) and where detailed records could be kept of the progression of the disease.

The first decision to withhold therapy has been defended on the basis that arsphenamine treatment had some serious side effects and was not a foolproof cure, although it cured many treated individuals. However, when penicillin became available to the public in 1947 and proved to be an effective and nontoxic cure for syphilis, the decision to continue to withhold therapy from the men was simply criminal. This experiment continued until 1972. By that time, 28 of the men had died of syphilis and 100 others had died of syphilis-related complications.

The study was dubbed the "Tuskegee experiment" because some of the laboratory facilities at Tuskegee University were used, but the study was designed and carried out by people working for public health agencies. When this experiment was discovered and made public in 1973, it provoked extensive congressional hearings, which formed the basis for current ethical rules governing the use of human volunteers. The U.S. government, after a long delay, offered free medical care to the men involved, but not to their infected wives or children, who were infected because of being born to syphilitic mothers. Grudgingly, health care was later extended to family members, but it took a lawsuit on behalf of the men to prod the government to offer a cash settlement, small by today's standards. To the end, the Public Health Service officials refused to acknowledge any wrongdoing and continued to defend the study on the basis that the men would not have had treatment anyway because of the lack of knowledge of medical services and their poverty, an argument that infuriated rather than convinced critics of the study. This incident is considered a tragic example of research abuse, racial oppression, and profound social injustice.

In May of 1997, President Clinton officially apologized for the gross injustice, but the Tuskegee Study has become a prime symbol of unethical research and governmental lack of concern about the fate of some of its most vulnerable citizens. This painful chapter in U.S. medicine had almost been forgotten—a fact that raised some serious questions about pretension to train students in bioethics—when it resurfaced in connection with the controversial AZT trial design. Critics of the AZT study invoked the Tuskegee precedent in their outrage over the fact that different standards of medicine were being applied to people in the developing world than to people in the developed world. Defenders of the AZT study pointed out that the women involved in the trials would not, under normal conditions, have received any therapy, but that defense was unacceptable to critics of the study design.

Source:

Jones JH. 1993. *Bad Blood: The Tuskegee Syphilis Experiment.* Simon and Schuster, New York, NY.

the future. A prospective study usually follows over time a group of human subjects (called a **cohort**), who may differ with regard to certain genetic or demographic traits, exposures, or treatments, to compare how those differences might impact disease outcome. An example of a prospective study is where the investigators design a trial to study the role of bacterial vaginosis in pregnant women as a risk factor for preterm birth (i.e., gestational age at birth of <37 weeks). In such a trial, two cohorts of 100 pregnant women each might be recruited, where one group consists of 100 women diagnosed with bacterial vaginosis at any time during weeks 16–24 of gestation and the other is a control group of 100 women without bacterial vaginosis during the same period of gestation. The pregnancy outcomes for all subjects are determined with regard to gestational age at birth. If the cohort with bacterial vaginosis during pregnancy shows a statistically significant increased rate of preterm birth over that of the control cohort, then that would indicate that bacterial vaginosis might be a risk factor for preterm birth.

Another type of human trial is called a "**retrospective**" **study,** which is often conducted on infectious disease outbreaks that have already occurred accidentally or naturally and that are studied in retrospect (looking back at past events) to obtain information about disease transmission or progression in humans. One example of a retrospective study is the case of a school bus driver with active tuberculosis (infected with *Mycobacterium tuberculosis*), who managed to infect a number of school children before his disease was diagnosed. This study, in retrospect, provided valuable information about various factors affecting transmission of tuberculosis. Retrospective study of this case revealed, for example, that the likelihood of infection was directly linked to the amount of time a child spent on the bus. The fact that

40 minutes a day versus 10 minutes a day made a discernible difference gave scientists a new appreciation of how infectious tuberculosis actually can be. Similarly, retrospective studies of foodborne disease outbreaks have helped to determine what types of people are most likely to develop a life-threatening disease in such cases.

Nonhuman Animal Models

For most studies of infectious diseases, nonhuman animals are the models of choice, when possible. Since the time of Koch and Pasteur, laboratory rodents have been the most widely used models for infectious disease research, because they are small and thus more easily (and cheaply) housed and cared for than larger animals, such as pigs or baboons. Although rodents are closely related to humans on the evolutionary scale, there are a number of important differences. Anatomically, they are similar to humans, except that they have fur and tails. But, there are also differences: humans have a single uterus, whereas mice and rats have a duplexed uterus (two chambers). Rodents also have a prominent cecum, whereas humans have a vestigial appendix. Unlike humans and mice, rats do not have a gallbladder. A fact that is frequently overlooked but could be a factor in the use of rodents to study intestinal disease is that rodents practice coprophagy (routine ingestion of one's own feces), whereas humans do not (except perhaps unintentionally via unwashed hands). Not surprisingly, rodents have very different gut, vaginal, and oral microbiota from humans.

There are undoubtedly many other differences, as is evident from the very different course some human diseases have in rodents. In some cases, a bacterial species related to, but different from, the pathogen isolated from humans can be found that causes similar disease in the animal model. For example, infections with a fish-specific *Mycobacterium* species causes similar disease symptoms in zebrafish as those observed in humans for *M. tuberculosis*, which causes tuberculosis. *Salmonella enterica* serovar Typhimurium, which causes diarrhea in humans, causes a systemic disease in mice that closely resembles the human systemic disease typhoid fever, which is caused by the human-specific *Salmonella* serovar Typhi. However, *S. enterica* serovar Typhi, which can be deadly in humans, does not infect mice. There are a number of other examples of this type of host specificity.

For certain diseases, scientists have developed more exotic animal models that may not mimic the human anatomy in many ways and may not mimic the disease in every respect, but still provide unique insights for certain aspects of the disease. Examples are ferrets as a model for gastric ulcers caused by *Helicobacter*, guinea pigs as models for tuberculosis, armadillos for leprosy (skin lesions caused by *Mycobacterium leprae*), chinchillas for otitis media (ear infections caused by *Haemophilus influenzae* or *Streptococcus pneumoniae*), and zebrafish for necrotizing fasciitis ("flesh-eating" disease caused by Gram-positive streptococci). The nematode *Caenorhabditis elegans* has been used as an animal model for certain pathogenic bacteria, such as *Pseudomonas aeruginosa,* that infect many different hosts. In fact, some scientists have been looking into plants as a model for infectious disease studies. An interesting, or at least entertaining, exotic-animal model is described in Box 8-2.

Ideally, an **animal model** for a human infection should contract a disease with symptoms and distribution of bacteria in the body that mimics what is observed during human infection. Similarly, animals should acquire the disease by the same route as humans. How far to trust an animal model that does not satisfy these criteria is a matter of judgment. Rather than establishing rigid criteria for whether an animal is a "good" or "bad" model for a disease, a variety of factors should be considered when choosing an animal model for human disease. Is it easy and inexpensive to maintain the animal? For some diseases, nonhuman primates are the only suitable animal models, yet these animals are difficult to obtain, particularly in large numbers needed to establish statistical significance, and are expensive to house and maintain. Another consideration is whether studies using the animal model have produced insights that are consistent with observations of the disease in humans. Has research on the animal model led to effective interventions in human disease? One of the main reasons for the failure of promising new vaccines and drugs is the lack of efficacy once the tests move from animal studies into humans. There are also ethical considerations. Does the model involve causing significant pain to the animal that might be considered unwarranted or unethical? This important topic is discussed further below.

More recently, another criterion has surfaced for choosing an animal model: the ease with which the animal model can be manipulated genetically. One of the primary reasons for putting forth the nematode *C. elegans* as an animal model, despite the fact that primitive worms are a long way evolutionarily from humans, is that there are many mutant strains of *C. elegans,* making it possible to investigate the effects of host genetic traits in the development of a disease. There are few scientists who would fail to agree that an imperfect animal model is better than no animal model at all. On the other hand, the course of diseases can be very different in distant models, such as *C. elegans*, that have innate immunity but lack adaptive immunity (see chapter 4).

Scientists are increasingly using modified conventional animal models, such as mice, to gain unique insights into particular infectious processes. Infant mice have immature immune systems compared to adult mice, and so they are often more susceptible to infection. Likewise, irradiated mice are immunocompromised because X rays have destroyed their immune cells; nude mice are genetically defective in their ability to produce T cells; neutropenic mice are defective in their ability to produce neutrophils; and SCID (severe combined immunodeficient) mice are genetically defective in their ability to produce B cells and T cells. All of these

Box 8-2.

A Blood-Curdling Animal Model

Despite repeated assurances from scientists that vampires do not exist, a segment of the public seems to place more credence in novels and movies than in the opinion of scientists and persists in holding onto a belief in vampires. Accordingly, a group of Scandinavian scientists decided to do the superstitious public a service (or perhaps pull its leg) by testing the old hypothesis that vampires can be repelled with garlic. Unfortunately for the scientists who wanted to conduct the studies, vampires are not easy to come by, so they had to resort, as scientists often do, to an animal model. They chose as a vampire substitute the blood-sucking, medicinal leech, *Hirudo medicinalis*.

Leeches are readily available because the medicinal leech has recently become respectable once again in medical circles due to its ability to prevent the formation of clots and internal accumulations of blood (hematomas). Hematomas and clots interfere with the healing of surgically reattached fingertips or ear segments because they block the circulation of blood to the reattached area. A person with a reattached fingertip or other limb has only to place a medicinal leech on that appendage for a short time every few days to eliminate clotting and hematoma formation. End result? Happy patient, happy leech!

The Scandinavian scientists borrowed a few leeches and set up an experiment in which leeches were given the opportunity to choose a garlic-smeared hand or a garlic-free hand for their next meal. The results of this experiment demonstrated that leeches actually preferred the garlic-smeared hand to a clean one. The difference was statistically significant. The scientists conducting this study modestly declined to extrapolate their results too recklessly, but their findings definitely raise questions about the efficacy of garlic as a vampire repellant. Does anyone think these scientists have a little too much time (not just garlic) on their hands? At least this study shows that, contrary to the usual portrayal in the media (especially movies), scientists do know how to have a little fun.

Source:

Sandvik H, Baerheim A. 1994. Does garlic protect against vampires? An experimental study. *Tidsskr Nor Laegeforen* **114**: 3583–3586.

types of mice are more susceptible to infection. Moreover, comparisons in different immunocompromised mouse models can lend insights into which immune responses are most important at different stages of infection caused by specific bacterial pathogens.

Another reason for remaining attached to the laboratory rodent as an infection model is the increasing number of available mouse strains with specific genetic alterations (i.e., **transgenic mice**). These include not only the so-called "**knockout mice,**" which have disruptions in specific genes, but also what we might call "**knock-in mice**," which have human genes introduced into their genome. Both knockout and knock-in mice have been used to study the attachment of *Helicobacter pylori* to the gastric epithelium. Knockout mice that lack **decay-accelerating factor (DAF)** have been used to demonstrate the binding of *H. pylori* to DAF, thus leading to inflammation of the gastric mucosa. Alternatively, a knock-in mouse, the Le^b mouse, which carries the human Lewis b antigen (one of the blood group carbohydrate antigens on the surface of human cells), has been used to study *H. pylori* infections, because the Lewis b antigen appears to be another receptor for attachment of *H. pylori* to gastric cells and gastric mucin. Such mutant animals offer vast new possibilities for research on infectious diseases.

The availability of transgenic animals is particularly important given the accumulating examples of specific genetic defects that predispose people to certain types of infectious diseases. For example, heterozygous carriage of the sickle cell trait confers partial immunity to malaria. Homozygous carriage of defective chemokine receptor genes confers resistance to HIV. Heterozygous carriage of the cystic fibrosis gene confers reduced severity of cholera diarrhea. Humans, particularly males, with a certain ε4 allele of the apolipoprotein E (ApoE) gene are more prone to atherosclerosis associated with *Chlamydophila pneumoniae* than those that do not possess this allele. Variant alleles of several complement proteins, such as properdin (also called factor P) and mannose-binding lectin (MBL), have been linked to increased childhood susceptibility to *Neisseria meningitidis* and other bacterial infections. As studies of human genetic disorders expand in scope, we will undoubtedly gain a better understanding of how human genetic variation affects patterns of susceptibility to bacterial infections. We will return to this topic again in chapter 10.

Two other useful animal models have emerged as important tools for studying infection processes and the host immune response. **Gnotobiotic animals** are raised in sterile or highly controlled environments, in which the microbes are either limited to only certain known microbes or excluded (**germ-free animals**). As a result, they have severely underdeveloped mucosa-associated lymphoid tissue (MALT) and no partial immunity due to prior exposure to microbes. Gnotobiotic and germ-free animals have been particularly useful for studying the development of immunity to a pathogen in a "truly naïve" (unimmunized) animal. Unfortunately, their immune systems are severely underdeveloped, and they are very expensive to buy and difficult to maintain. **Specific-pathogen-free (SPF) animals** are raised in an environment free of a particular pathogen, but are otherwise exposed naturally to other microbes. These SPF models eliminate preexisting immunity due to prior exposure to a particular microbe that might complicate interpretation of host response to the pathogen of interest. These animals have provided invaluable insight into the nature of commensalism and the characteristics that determine whether a microbe will become a commensal or a pathogen.

Measuring Bacterial Infection in Animal Models

Ethical Considerations

As was the case for human studies, experiments involving animals involve numerous ethical issues. Disease models invariably involve infection of animals, most often rodents, with bacteria that cause disease symptoms, pain, distress, and sometimes death. Therefore, there must be truly compelling reasons for carrying out these experiments. The genetic and physiological properties of the bacterial strains to be tested should be fully characterized before animal experiments are considered. The animal experiments should be designed to test critical hypotheses that can provide useful information for understanding the bacterial disease being studied, even if negative results are obtained. Importantly, all procedures used in animal experiments funded by the federal government must be approved in advance by a duly appointed institutional committee, called an **Institutional Animal Care and Use Committee (IACUC)**, whose members have expertise and experience performing animal experiments, including veterinarians. Unlike many other types of research, even if you have a great idea, you need to get the procedure approved in advance before performing an experiment involving the use of an animal.

Approved animal protocols contain a variety of important details about the experiments to be performed and must conform to stringent standards of experimental design. The researchers need to argue persuasively that no alternative model can provide equivalent information to that obtained from using the proposed animal model. The protocol must contain extensive information about the choice of animal model to be used; ways to minimize the number of animals required that will still give statistically significant results; precedents from the scientific literature; and documentation of appropriate animal use and safety training of laboratory personnel. Besides detailed experimental methods, these documents must contain extensive information about the care of the animals before and during the experiments, anesthesia and minimization of pain and discomfort, and euthanasia. In general, death of the animals is rarely used as an endpoint, and complete monitoring rubrics to judge pain and discomfort must be compiled, including how to judge whether the animals are sufficiently sick or moribund to require euthanasia. Finally, these protocols must be reviewed by the IACUC and renewed annually.

Animal Model Basics

Because virulence factors are defined as factors that allow a bacterium to survive in the host, to colonize and establish infection, to cause symptoms, and sometimes to cause death, measuring properties such as infectivity and lethality is an important part of virulence studies. There are several guiding principles to the design and execution of animal models of infectious disease. First, an animal species that reflects the disease process in humans or that will answer the questions being asked should be chosen. There must be a truly compelling rationale to use species higher than rodents or zebrafish. Next, a route of infection must be chosen. Often, bacterial broth or plate cultures are diluted to defined starting doses based on numbers of bacteria in the initial inoculum, usually reported as **colony-forming units (CFUs**). Other sources for starting bacterial cultures are also used, such as planktonic bacteria released from biofilms.

In the simplest models of systemic infection, the bacterial dose is simply injected with a syringe subcutaneously, intramuscularly, or into the tail vein or the peritoneal cavity of a mouse. In simple models of invasive respiratory infection, a small drop containing the bacteria is placed on the nose of an anesthetized mouse whose mouth is gently held closed for a moment. The mouse will then inhale the drop into its nasopharynx and lungs. In other simple models of infection, the dose of bacteria is delivered orally or directly to the lung or stomach of anesthetized animals through fine tubes or specialized syringes. Other, more complicated modes of infection used for specific models of disease are available but not discussed here.

Once the animal species and route of infection are chosen, methods for monitoring the infection must be chosen and planned. In some cases, very tiny amounts of blood are removed from tail veins at different intervals, and the number of CFUs in the blood is determined by spreading dilutions onto plates. Bacteremia can lead to the accumulation of up to 10^8 to 10^9 CFUs per mL of blood. However, in many cases, the number of CFUs per organ needs to be determined, and this paradigm involves euthanizing the animal, removing the organ, grinding it in physiological saline solution, and spreading a dilution onto plates to determine the number of CFUs in the sample. To determine colonization in the nasopharynx, saline can be injected into the dissected trachea of the mouse and then re-collected at the nose, diluted, and counted. In all of these models, several animals need to be sacrificed for each dose and time point following infection to obtain statistically significant results, and the number of animals can become quite high. New methods to image infections in live mice, such as **biophotonic imaging**, which substantially reduce the number of animals needed for some of these experiments, are described below.

Survival Curve Analysis and Biophotonic Imaging

Survival curve analysis is a commonly used approach that determines the median survival time of animals following infection with a wild-type bacterial pathogen and mutants that are being tested for their virulence properties. Advantages of this approach are that it can provide statistically robust results using a relatively small number of animals and that it can be combined with biophotonic imaging. In the example shown in Figure 8-1, a wild-type strain of *S. pneumoniae* and a Δrel_{Spn} mutant containing a deletion of the gene, whose protein product catalyzes the synthesis of the signaling molecule alarmone (a mixture of guanosine pentaphosphate and guanosine tetraphosphate [(p)ppGpp]), were inoculated intranasally at a dose of about 10^7 CFUs into a specific strain of outbred mouse. A control was included, in which the Δrel_{Spn} mutation was complemented by a copy of the wild-type rel^+_{Spn} gene located elsewhere in the bacterial chromosome. Ten mice were inoculated per strain tested. The infected mice were examined for moribundity every few hours following inoculation. As noted above, death was not used as an endpoint, but rather, mice that were moribund (i.e., showing obvious signs of acute illness) were euthanized before death occurred.

The survival curve (Figure 8-1A) was generated by counting the moribund and non-moribund mice at each time point. A special kind of statistics, Kaplan-Meier analysis with log-rank tests, was used to generate the median survival times and the *P* values, which are an indication of statistical difference. The survival curve showed that complemented and wild-type bacteria caused statistically similar median survival times of about 60 hours at this dosage in this mouse strain. In contrast, the median survival time of mice inoculated with the Δrel_{Spn} mutant was about 140 hours, which means that the virulence of the Δrel_{Spn} mutant was significantly attenuated. Another important lesson from this study is that median survival time depends on the bacterial dose and the type of mouse used.

But what of the infection? We could have removed a tiny drop of blood or removed the lungs to determine the number of CFUs at each time point of the experiment. However, the load of the bacteria in the blood would probably not be that informative, because the blood CFU count tracks with severity of infection, and moribund animals are bacteremic. To determine the number of CFUs per lung per strain, we would need at least five animals per time point, which would increase the number of animals needed from 10 mice per bacterial strain tested to about 100 mice per strain. Although both of these approaches are legitimate and would yield meaningful results, new technologies have made other approaches that require fewer animals possible.

One new alternative approach is biophotonic imaging. In this approach, a bacterial luciferase operon (*luxABCDE*) isolated from a *Vibrio* species is introduced at a chromosomal location in the bacterial strains to be tested that does not affect virulence of the bacterium being tested. This luciferase operon contains all of the genes for making bacterial luciferase and its substrate so that the bacteria will glow in the dark. Mice are then infected with the bacteria carrying the luciferase operon as described above. However, now, at

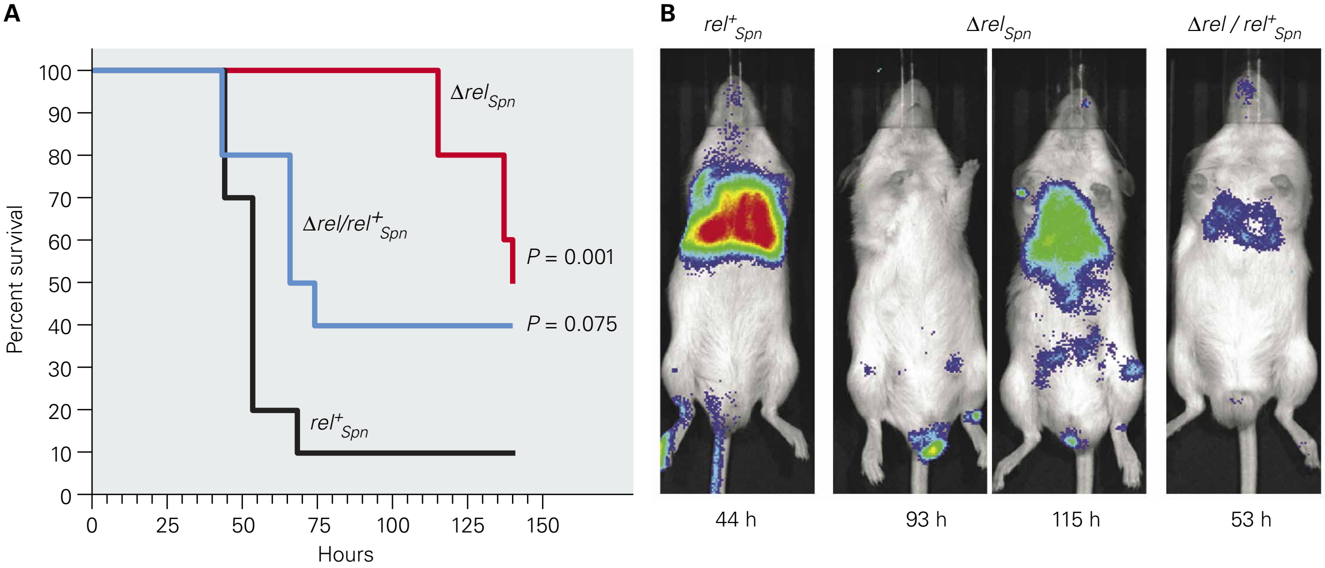

Figure 8-1. Disease progression and survival of mice infected with *luxABCDE* rel^+_{Spn}, *luxABCDE* Δrel_{Spn}, and complemented *luxABCDE* Δrel_{Spn} *bgaA*::P_{rel}-rel^+_{Spn} strains of *Streptococcus pneumoniae*. Mice were inoculated intranasally with 6×10^6 CFUs and disease progression was followed in real time by (**A**) survival curve analysis and (**B**) biophotonic imaging. rel^+_{Spn} infected mice develop lung infections after 44 h, while mice infected with Δrel_{Spn} do not develop lung infections until 115 h. When mice are infected with bacteria where the Δrel_{Spn} is complemented, lung infections can be seen developing by 53 h. Adapted from Kazmierczak KM, Wayne KJ, Rechtsteiner A, Winkler ME. 2009. *Mol Microbiol* **72:**590–611, with permission.

intervals of about 8 hours, the mice are not only checked for disease progression, but are also lightly anesthetized in a dark chamber connected to a supersensitive digital camera that can detect and quantitate light produced by the bacterial infection inside of the mouse. The camera literally looks right through the mouse to follow the course of infection (Figure 8-1B). In the example shown, wild-type and complemented strains of *S. pneumoniae* caused localized pneumonia in the lungs of the mice. However, the Δrel_{Spn} mutant changed the course of infection completely in most of the mice. The bacteria initially localized to the lower abdomen, probably in lymph nodes, instead of the lungs. Later, the bacteria move to the peritoneal cavity before moving to the lungs and bloodstream. Thus, the rel_{Spn} gene is not only required for full virulence, but it also dictates the normal course of infection by *S. pneumoniae.* Interestingly, new insights into the disease process were discovered using this approach, since it would not have been possible to detect this change in the course of infection by simply dissecting the lungs. And, the experiment required far few animals!

ID_{50} and LD_{50} Values

Two other common measures used widely to define virulence in animal models are the **50% lethal dose (LD_{50})** and **50% infectious dose (ID_{50})** (Figure 8-2). The **LD_{50} value** is the dose at which 50% of the animals are moribund (i.e., the number of bacteria needed to cause terminal acute infections in 50% of the animals). LD_{50} values measure a much later event in the disease process; namely, moribundity of the host that will lead to eventual death. But, not all pathogens kill the host, even during a natural infection. In such cases, it is necessary to have some other means of measuring the extent of infection caused by the bacterium. The **ID_{50} value** is the infectious dose at which 50% of the animals are infected (i.e., the number of bacteria necessary to infect 50% of the animals exposed to the bacterium). ID_{50} values measure the ability of the bacterium to colonize the host, establish infection, and manifest measurable disease symptoms. The most common method used for determining an ID_{50} value is to determine the number of CFUs present at a certain site in the animal (e.g., blood, liver, spleen, lung, lymph node, or brain) after a certain incubation time. There are other disease indicators that can also be used, such as the host's temperature (fever), the size of necrotic lesions that form, the degree of swelling from edema, or the extent of loss of motor function over time. Biophotonic image analysis, instead of CFUs, can also be used to follow the progress of an infection for ID_{50} value determinations.

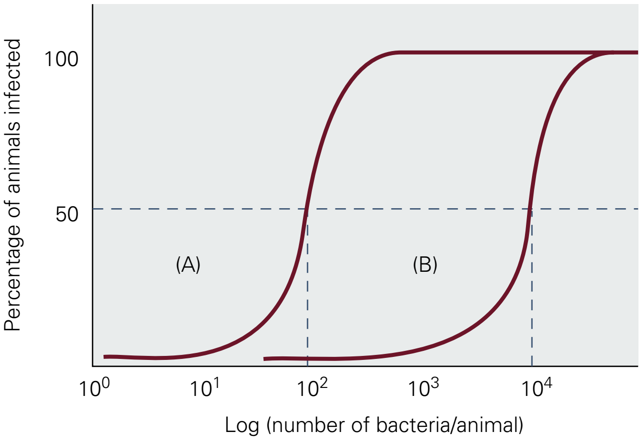

Figure 8-2. Typical curves obtained when determining ID_{50} values for bacteria with different levels of infectivity. For bacterium A, only 100 cells per animal are required to cause disease in 50% of the animals ($ID_{50} = 10^2$). In comparison, for bacterium B, 10,000 cells per animal are required to cause disease in 50% of the animals ($ID_{50} = 10^4$). Therefore, bacterium A is more infectious than bacterium B because it requires fewer cells of bacterium A to cause disease.

Plots of the number of animals infected (or that become moribund) versus the number of bacteria present in the initial inoculum (dose) are sigmoidal, not linear (see Figure 8-2). To locate the 50% point precisely, standard statistical methods, such as the procedures of Spearman-Karber, Reed-Muench, or probit analysis, are used. These methods not only determine the 50% point mathematically, but also provide a measure of experimental error that is important for deciding whether two ID_{50} or LD_{50} values differ from one another by a statistically significant amount. The reason a 50% value is determined rather than a 100% value is that it is much easier to determine a 50% value accurately, because it is in a region of the curve where maximal change occurs. The lower the LD_{50} or ID_{50} value, the more lethal or infectious, respectively, the bacterium is.

ID_{50} and LD_{50} values have proven to be useful measures of infectivity and lethality, but they have some important limitations. These parameters reflect the cumulative effect of the many steps involved in colonization and production of symptoms. Therefore, they lack a certain level of sensitivity or resolution detail, and the failure of a mutation in a bacterial gene to increase the ID_{50} or LD_{50} value does not always mean that the mutation did not affect some virulence determinant. Moreover, ID_{50} and LD_{50} determinations require the use of a large number of animals, usually 5 to10 at each bacterial dose.

To determine the role of a particular virulence factor in a pathogen that has multiple virulence factors, it is sometimes necessary to eliminate one virulence factor genetically in order to be able to determine the role of a second virulence factor. Once the first virulence factor is eliminated, one can compare the effect on virulence of deleting the second virulence factor to the virulence of the attenuated strain lacking the first virulence factor. However, difficulties can be encountered in attempting to study highly attenuated pathogens, including opportunistic pathogens. In these cases, large numbers of bacteria (more than 10^9) must be injected into the animal model. Because the ID_{50} or LD_{50} value is already so high and such large numbers of bacteria are already needed to observe virulence, it is difficult to see the effects of mutations that might affect virulence by looking for increases in the ID_{50} or LD_{50} value in these cases.

When generating the data for ID_{50} or LD_{50} plots, it is important to define clearly what the measurement is that is being determined. For example, infectivity might be defined by CFUs detected in the spleen of a mouse after three days of infection, but for highly attenuated mutant strains, it may be necessary to determine the CFUs after five or more days of infection. But, the ID_{50} value determined using this definition of infectivity might differ significantly from one in which the bacteria are counted after five days of infection. Likewise, the LD_{50} value that is determined as the number of moribund animals after three days most likely will differ from the value determined after five days. In addition, it is extremely important to remember that these infection parameters will depend on the animal model used. In some cases, outbred mice with robust immune systems are used, and the response to infection may vary greatly from animal to animal. In other cases, animal models are chosen to give the bacteria as much of an edge as possible, so that infection can be obtained reproducibly in most or all of the animals. A trait that might be critical to establishing an infection in an animal model, where the animal was chosen for its high susceptibility, might not be as critical in the more challenging environment of the body of an immunocompetent human.

Another limitation of ID_{50} and LD_{50} values is that they at best indicate the relative virulence of strains of the same bacterium causing the same disease, but they can be misleading when misused to compare bacteria causing different diseases. For example, the bacterium that causes cholera (*Vibrio cholerae*) has an ID_{50} value of about 10^4 bacteria when ingested by humans, while the bacterium that causes bacterial dysentery (*Shigella dysenteriae*) has an ID_{50} value of 10–20. At first glance, this might suggest that dysentery leads to a more serious disease; however, this is not the case. In fact, cholera is often a fatal disease, whereas bacterial dysentery seldom causes death. The difference in ID_{50} values is primarily due to the relative ability of the two different bacterial species to survive passage through the acid environment of the stomach after being ingested.

Competition Assays

A way to make infection experiments more sensitive is to use a competition assay and to determine the **competitive index (CI)**. The CI is defined as:

$$CI = \frac{output\ ratio \left(\dfrac{CFU\ mutant}{CFU\ wild\ type} \right)}{input\ ratio \left(\dfrac{CFU\ mutant}{CFU\ wild\ type} \right)}$$

In this assay, the animal is infected with a mixture of mutant and wild-type bacteria at a particular input ratio (CFU mutant/CFU wild type). After allowing the bacteria time to establish an infection, samples are taken from various parts of the animal and the number of mutant to wild-type bacteria are counted to give an output ratio (CFU mutant/CFU wild type). If the ratio of mutant to wild-type bacteria is the same as in the infecting dose ($CI = 1.0$), then the mutation had no detectable effect on colonization and survival in the host. However, if the wild type outcompetes the mutant ($CI < 1.0$), then the mutation clearly had a detrimental effect on colonization (and usually also a negative effect on virulence). If the mutant outcompetes the wild-type bacteria ($CI > 1.0$), then the mutation caused an increase in colonization and survival in the host. One reason this type of assay is more sensitive than using ID_{50} or LD_{50} values is that the mutant and wild-type bacteria are competing for the same turf, so we are comparing the fitness of the mutant to compete with the wild-type bacterium for survival in the host environment. A second reason is that it is often easier to quantify the ratio of the wild type to mutant bacteria more accurately than it is to determine the ID_{50} or LD_{50} value accurately. Importantly, competition assays allow investigators to use far fewer animals, since it is not necessary to use multiple animals for each dose tested to generate a dose curve.

However, there are some serious issues to consider when interpreting competition experiments. Mutants that grow more slowly in broth cultures will likely lose in competition assays in an animal. But, this does not mean that the process affected in the mutant is necessarily contributing directly to the virulence process. It can also be difficult to interpret what subtle differences obtained in competition experiments actually mean in terms of the mechanism of virulence. The competition experiment paradigm precludes the use of some detection approaches, such as biophotonic imaging, because the light produced from the parent or mutant is indistinguishable. A serious concern is *trans*-effects that may allow mutants defective in virulence to grow in the presence of the wild-type parent strain. For example, suppose a mutant cannot produce a secreted toxin that is required for disease symptoms. Using single-strain approaches, such as survival curve or LD_{50} curve analysis, the parent strain would appear as virulent, whereas the toxin mutant would be strongly attenuated. But, in the competition experiment, the parent strain may secrete sufficient toxin for the mutant to grow fully. In this case, approximately equal CFUs of the parent and mutant would be recovered from the infection, and the conclusion from the competition experiment would be that the mutant was fully virulent. This is clearly the wrong conclusion. Usually a combination of single-strain and competition infection experiments is needed to begin to understand processes as complicated as bacterial virulence in animal models.

Tissue Culture and Organ Culture Models

Tissue Culture Models

Although animal models are the "gold standard" of research on bacterial virulence, animals present a complex system in which many variables cannot be controlled. Cultured mammalian cells are commonly used to provide a more easily controlled system for investigating host-bacterium interactions. Tissue culture cells can be grown in defined medium under reproducible conditions with only one or a limited number of cell types represented, which makes measurements and interpretations easier and more reproducible. It is also easier to perform experiments involving radioactive compounds and to introduce foreign DNA into tissue culture cells. Cultured cells can be readily visualized by microscopic techniques, and cells expressing certain fluorescently labeled marker proteins can be sorted using high-speed devices. They also cost less per day to house than laboratory rodents, do not fight with each other, and have seldom been known to escape from their cages. Because of the very important role that tissue culture cells have played in molecular investigations of bacterium-host cell interactions, it is important to understand their limitations, which must be kept in mind when interpreting results of experiments.

Primary cultures of mammalian cells that are not derived from tumors can be obtained from animal tissue, biopsy material, or the blood of human volunteers (e.g., macrophages), but these primary cell types usually undergo a limited number of divisions in culture and can only be maintained for a couple of weeks. Consequently, "immortalized" cells that continue to divide in culture are often used in routine experiments. These immortalized cells are derived directly from a tumor, by fusing a primary cell type with a tumor cell, or by continuous selection of primary cell lines for unregulated growth. Propagation of mammalian cells that are unregulated for growth in culture leads to the accumulation of numerous mutations, gene rearrangements, and gene duplications. These mutations are not only uncharacterized because they are so numerous and complex, but they are also not easily reproduced. Two separate sets of primary cells from the same source that are treated in exactly the same way to produce immortalized cell lines will have different combinations of mutations and rearrangements. Another way of immortalizing the cells is to introduce certain viral proteins into them. Thus, not only are cultured cells far from being genetically identical to cells in the tissue or organ from which they were derived, but different lines developed from the same type of cell are not necessarily identical. Cell lines that are passed repeatedly in culture will continue to develop new mutations. Therefore, the fact that two different investigators are using a cell line with the same name does not necessarily mean that they are using genetically identical cell lines.

Cells in culture are no longer in the same environment as in the organ of origin, and many genes that were expressed by cells in an intact organ may not be expressed in cultured cells. Culture medium does not represent the natural conditions and biochemical composition found in the body, where fluids and cellular secretions may significantly influence cellular processes in ways that are not reproducible in a culture system. Consequently, tissue culture cell lines lose many traits of the original tissue from which they were derived and may not display the same surface markers in culture. To make the transition from a differentiated, nondividing cell to a rapidly dividing cell, cultured cells must be stripped of many of the properties that made them the type of cell they were in the first place. A consequence of this is that bacterial and viral pathogens that are highly specific for a particular tissue or cell when causing an infection in an intact animal are frequently able to invade cultured cells derived from tissue they do not normally infect. This may be due to expression of different receptors on the cell surface. These differences in gene expression can be corrected to some extent by addition of growth stimulants or hormones to the culture medium or by providing an artificial **matrix** (also called a **substrate**) for the cells to grow on, but no one has yet managed to make an immortalized cell line into a completely faithful replica of the same cells in the body.

A related problem is that most cultured cells lose their normal shape and distribution of surface antigens. Cells in an intact animal are usually **polarized**. That is, different regions of the cell surface membrane are exposed to different environments (e.g., lumen, adjacent cells, underlying blood, and other tissue types). Membranes on different sides of polarized cells contain different sets of proteins, a feature that is presumably important for their function. This is illustrated in Figure 8-3 for a layer of mucosal cells, but the same considerations apply to cells in other parts of the body. In a layer of normal mucosal cells, the **apical surface** is exposed to the external environment (e.g., the lumen contents in the gastrointestinal tract), whereas the **basal** and **lateral surfaces** are in contact with the **extracellular matrix** (a combination of proteins and polysaccharides that "glues" the cells together). Mucosal cells in the gastrointestinal tract and some other tissues are also connected to each other by **tight junctions**, which are made of specialized tightly binding protein complexes that make an impermeable connection between adjacent cells.

In contrast, tissue culture cells that are grown as nonconfluent monolayers do not have differentiated surfaces, and proteins that are found only on the apical or on the basal-lateral surfaces of cells *in vivo* may be distributed over the entire surface of such cells, assuming they are produced at all. Allowing the cells to grow to confluence does not necessarily solve this problem. It is usually necessary to provide an extracellular-matrix substitute and to provide

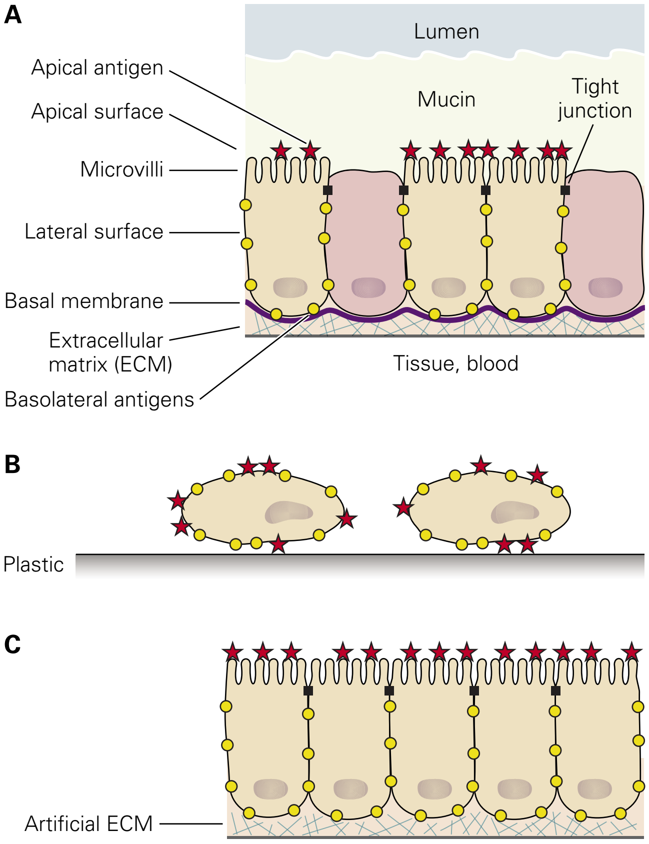

Figure 8-3. Differences between cells of an actual mucosal membrane and tissue culture cells. (**A**) Actual membrane *in vivo*. (**B**) Nonconfluent, nonpolarized tissue culture cells. (**C**) Polarized monolayer of tissue culture cells attached to a semipermeable membrane.

hormones to obtain a polarized monolayer in culture. Production of a polarized monolayer in culture has been achieved for some types of cell lines, but the expression and distribution of relevant surface molecules should still be checked before concluding that a polarized cell monolayer in culture is the same as tissue in the intact animal. In practice, this is seldom done.

Still another problem with using cultured cells as a model is that human mucosal surfaces are covered with mucus and bathed in solutions that are difficult to mimic in an *in vitro* system. The fluid bathing the small intestinal and colonic mucosal cells is anaerobic and contains bile salts. The fluids bathing the vaginal mucosal cells and the bladder mucosal cells also have a low oxygen content and high concentrations of metabolites, such as urea or lactic acid, that could have an effect on mucosal cell physiology. Finally, real tissues consist of specific arrangements and proportions of multiple cell types that are not modeled by cell monocultures. Co-culturing more than one cell type has been done for a number of systems to mimic the natural interactions that might occur. For example, adding activated T cells or B cells, which serve as cellular sources of cytokines and chemokines, to a culture of dendritic cells can induce them to undergo maturation. However, exactly mimicking host cell-cell interactions in tissue culture remains problematic in many cases.

The fact that there are problems with existing tissue culture cell lines does not mean that such cell lines are not extremely useful. If their limitations are kept in mind, cultured cell lines can be marvelous tools for discovery. One recent example of the use of cell culture to address an important issue concerns formation of biofilms by the respiratory pathogen *S. pneumoniae*. Unlike many other bacterial pathogens (e.g., *P. aeruginosa*), encapsulated *S. pneumoniae* strains do not readily form biofilms on abiotic surfaces, such as plastic or glass. This property hindered the study of the role of biofilm formation in colonization by *S. pneumoniae*. Recently, it was shown that *S. pneumoniae* cultured on human respiratory epithelial cells forms highly ordered, antibiotic-resistant biofilms similar to those observed in animal colonization models. Furthermore, certain conditions that mimic host infection, such as increased temperature (e.g., a fever), lead to dispersal of virulent, planktonic *S. pneumoniae* cells from biofilms formed on host epithelial cells. In general, once a new phenomenon has been discovered in cultured cells (e.g., attachment of bacteria to a mammalian cell receptor or reorganization of host cell cytoskeleton), experiments can be designed that use organ cultures or animals to test the importance of the phenomenon *in vivo*. It is not uncommon for a mutation that affects the ability of a bacterium to infect a tissue culture cell to have no effect when tested in animals, so taking the study from tissue culture cells to the animal is an important step. In other words, tissue culture cells can be important for generating hypotheses that need to be tested in intact animal models. It is often difficult, however, to take the results obtained from studies of tissue culture cells and extrapolate them directly to the disease in humans.

Gentamicin Protection Assay for Cell Adhesion and Invasion

Tissue culture cells have been widely used to study adherence properties of bacteria and are particularly useful for identifying virulence factors involved in binding and invasion of host cells by intracellular pathogens. One such assay that researchers frequently use to distinguish between mutants that are defective in adhesion (attachment to the cell surface) from those that are defective in invasion is the **gentamicin protection assay** (Figure 8-4). In this assay, a culture (or monolayer) of mammalian cells is incubated with bacterial cells at a certain **multiplicity of infection (MOI)**, which is defined as the number of input bacterial cells per mammalian cell that are present in the culture tube or well of a culture plate. Following incubation for a period of time to allow binding and invasion to occur, which will vary depending on the particular organism being examined and

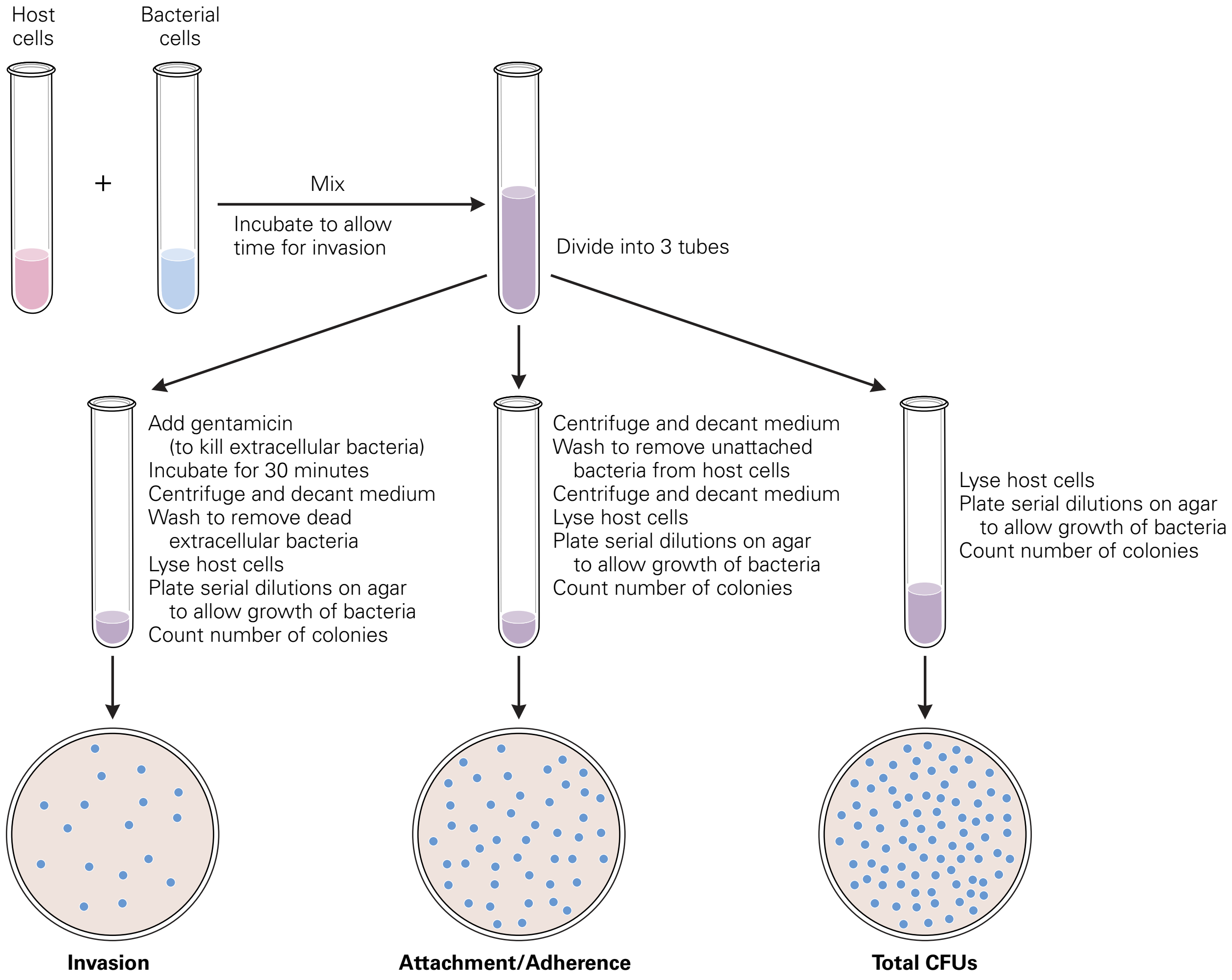
Host cells
Bacterial cells
+
Mix
Incubate to allow time for invasion
Divide into 3 tubes
Add gentamicin (to kill extracellular bacteria)
Incubate for 30 minutes
Centrifuge and decant medium
Wash to remove dead extracellular bacteria
Lyse host cells
Plate serial dilutions on agar to allow growth of bacteria
Count number of colonies
Centrifuge and decant medium
Wash to remove unattached bacteria from host cells
Centrifuge and decant medium
Lyse host cells
Plate serial dilutions on agar to allow growth of bacteria
Count number of colonies
Lyse host cells
Plate serial dilutions on agar to allow growth of bacteria
Count number of colonies
Invasion
Attachment/Adherence
Total CFUs

Figure 8-4. **Gentamicin protection assay.** Shown are the modified steps in a gentamicin protection assay for mammalian cells that are grown in suspension.

the number of receptors present on the mammalian cell surface, the samples are divided into three tubes or plate wells.

For the first tube, which will provide the total number of bacteria present (CFUs) in the tube (or well) of mammalian cells, the medium containing unattached bacterial cells and the mammalian cells are centrifuged to collect all bacterial and mammalian cells and the cell-free medium is removed. The pellet is resuspended in buffer with mild detergent or with gentle scraping to lyse the mammalian cells (these disruption methods lyse the mammalian cells, but do not lyse the bacteria). The resulting suspension of bacteria is serially diluted and plated on agar plates, and the bacteria are allowed to grow to form colonies, which are counted. This represents the total number of CFUs in each tube (or well) at the end of the experiment. For the second tube, which will provide the total number of adherent (attached) bacteria (CFUs), the infected mammalian cells are centrifuged and the pellet is first washed with a buffered solution several times to remove any nonadherent (unattached) bacteria. The mammalian cells with adherent bacteria are then lysed, and serial dilutions of the suspension are plated to determine the number of cell-associated bacteria (denoted as adherent CFUs). The ratio of cell-associated CFUs to total CFUs at the end of the experiment is defined as the **adhesion frequency**.

For invasion assays, a third tube is centrifuged and washed as above to remove nonadherent bacteria, and fresh culture medium containing the antibiotic **gentamicin** is added to the tube and the mammalian cells are gently resuspended. Gentamicin cannot enter mammalian cells, so it kills only extracellular bacteria and does not kill bacteria that have already entered the mammalian cells (i.e., internalized bacteria are protected from the antibiotics and therefore survive and can be counted). The cells are incubated with the gentamicin-containing medium for anywhere from 30 minutes to 2 hours, at which time the culture is centrifuged and the medium is removed. The mammalian cells are washed to remove unattached bacteria and mammalian cells are lysed and the bacteria resuspended, as described previously. Serial dilutions of the bacterial suspensions are then plated to determine the number of intracellular bacteria (denoted as gentamicin-resistant CFUs). The ratio of gentamicin-resistant CFUs to adherent CFUs at the end of the experiment is defined as the **invasion frequency**. This process allows invasion to be measured as a separate event from adherence. It is possible to report the number of gentamicin-resistant CFUs to total number of CFUs in the well, which may be very different depending on the frequency of adherence and growth rate of a given bacterial strain for the given mammalian cells used. There may also be some variation in the details of the experiment. For example, the cells might form adherent monolayers in culture instead of growing in suspension, requiring some adjustments in the washing, centrifuging, and lysis steps.

If a bacterial mutant is defective in an adhesion factor that prevents it from attaching to the mammalian cell, then no colonies will form on the agar plate for the second and third sets of tubes or wells. Mutants that can still adhere but are defective in invasion factors, so that they cannot enter mammalian cells or survive once inside, will produce colonies on plates from the second set of tubes or wells, but will have no colonies on plates from the third set of tubes or wells. By comparing the adherence frequencies and the invasion frequencies for wild-type bacteria versus mutants, it is possible to determine the nature of the virulence factor that was defective in the mutant bacteria.

Studying pathogenic mechanisms of *Campylobacter jejuni*, a foodborne pathogen that causes diarrhea, is hampered by the lack of simple animal models that mimic human disease, so cell culture assays have provided useful alternative ways to investigate *C. jejuni* interaction with host epithelial cells. Use of the gentamicin assay has shown that invasiveness varies considerably depending on the *C. jejuni* strain, as well as the human cell lines used, the number of bacteria in the inoculum (MOI), and other assay conditions. Researchers in the field have consequently sought to standardize assay conditions and to set up tests for comparisons between strains. One such test is to generate an **invasion success curve** (Figure 8-5), which plots the log(MOI/cell) versus the log(invaded bacteria/cell) to give the minimal MOI (minMOI) required to obtain the maximum number of internalized bacteria per mammalian cell (BI_{max}). In comparing strains, those with lower minMOIs are more invasive.

Plaque Assay for Intracellular Survival and Cell-to-Cell Spread

Some intracellular pathogens, such as *Legionella* and *Chlamydia*, are able to invade eukaryotic cells, multiply within the eukaryotic cell until they burst from the host cell, and then spread from one host cell to the adjacent host cell. During this process (called **cell-to-cell spread**), the bacteria replicate within each invaded host cell, killing the host cell in the process and creating a cleared zone of killed cells (called a **plaque**) around the initial cell that was invaded. Some intracellular pathogens, such as *Listeria* and *Shigella,* are also able to spread laterally within a monolayer by propelling themselves into the adjoining cell without being released into the medium.

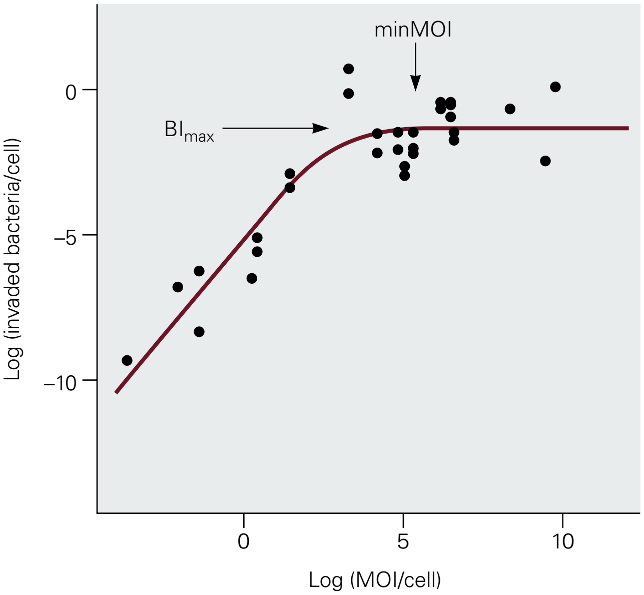

Figure 8-5. Invasion success curve. Shown is an invasion success curve for an invasive strain of *Campylobacter*. The average number of invaded bacteria per mammalian cell was calculated for each well in a plate over a wide range of MOIs, starting with a low MOI and relatively small numbers of internalized bacteria until a maximal invasion plateau (BI_{max}) is reached, where increasing the MOI no longer increased the number of internalized bacteria. Each data point represents the results obtained for one plate well. The BI_{max} and minMOI (lowest MOI required to reach the BI_{max}) values are indicated by arrows. In comparing bacterial strains, those with lower minMOIs are more invasive. Note that in order to more easily observe the effect, a log-scale is used to generate the curve. Reprinted from Friis LM, Pin C, Pearson BM, Wells JM. 2005. *J Microbiol Methods* **61:**145–160, with permission.

A modified gentamicin tissue culture assay that allows for assessment of an intracellular pathogen's ability to spread from cell to cell is the **plaque assay** (Figure 8-6). In this assay, tissue culture cells are grown on plates to form an even confluent monolayer and bacteria are added to the medium and incubated with the mammalian cells for a short period of time to allow invasion to occur. The medium with unattached bacteria is removed and the monolayer is incubated with gentamicin to kill any remaining extracellular bacteria. The monolayer is then gently covered with another layer of soft agar containing gentamicin (to prevent diffusion of the bacteria through the medium) and the cells are further incubated. After a while, the living cells are stained and plaques (cleared areas) in the monolayer can be observed where bacteria have invaded and spread from cell to cell, killing the mammalian cells in the process. Mutants

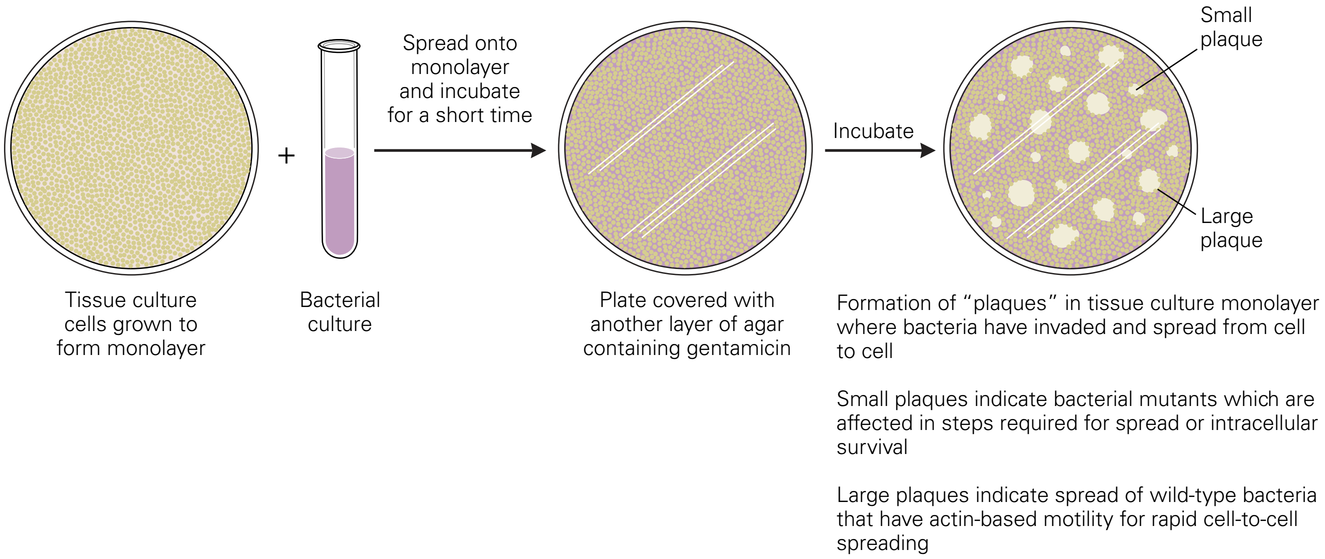
Spread onto monolayer and incubate for a short time
Incubate
Small plaque
Large plaque
Tissue culture cells grown to form monolayer
Bacterial culture
Plate covered with another layer of agar containing gentamicin
Formation of "plaques" in tissue culture monolayer where bacteria have invaded and spread from cell to cell
Small plaques indicate bacterial mutants which are affected in steps required for spread or intracellular survival
Large plaques indicate spread of wild-type bacteria that have actin-based motility for rapid cell-to-cell spreading

Figure 8-6. **Plaque assay for assessing intracellular survival and replication and cell-to-cell spread by an intracellular pathogen.** Shown are the steps taken for determining whether a bacterium that enters or invades a mammalian cell can survive, replicate, and subsequently lyse the host cell and spread to adjacent host cells, resulting in clearing of the host cell monolayer to form cleared zones with no host cells (plaques). Intracellular bacteria that are able to propel themselves through actin-based motility into adjacent cells via cell-to-cell spread form large plaques. Intracellular bacteria that can still replicate in the host cell, but lack this actin-based motility, form small plaques. Bacteria lacking factors needed for intracellular survival or replication form no plaques.

that are defective in efficient cell-to-cell motility will form small plaques, while mutants that are defective in factors necessary for invasion and/or intracellular survival or replication do not form any plaques.

Fluorescence Microscopy Techniques for Assessing Effects of Pathogens on Host Cells

The development of new reagents for probing the interior of a mammalian cell or for changing its chemical environment has generated opportunities for sophisticated new approaches to study bacterium-host cell interaction. For example, fluorescent dyes attached to specific reagents (chemicals, proteins, or monoclonal antibodies) that bind specifically to host cell cytoskeletal components, such as actin, have been used to follow by fluorescence microscopy the cytoskeletal rearrangements caused by bacteria attaching to and invading host cells. These methods have also been used to observe the changes in cellular morphology caused by toxins or secreted effector proteins produced by bacteria.

One particularly useful reagent for visualization of cytoskeletal rearrangements caused by the formation of actin filaments in cells is the marine metabolite **phalloidin**, which is a compound that binds very tightly to polymerized F-actin (which forms the actin filaments), but not free monomeric G-actin. When phalloidin is linked to a fluorescent dye, such as **rhodamine** (red) or **fluorescein isothiocyanate** (green), fluorescence microscopy can be used to monitor actin cytoskeletal changes and actin stress fiber formation (Figure 8-7). Calcium release from intracellular stores (called **calcium mobilization**) is another intracellular response often triggered by interactions of a cell with bacteria or bacterial toxins. A number of fluorescent dye reagents (such as **Fura-2**) that can detect calcium levels inside tissue culture cells are available.

A number of host cellular markers have been developed that allow researchers to visualize where the bacteria or bacterial proteins are located inside the host cells. These cellular markers are mammalian proteins that are known to localize to particular compartments within the host cell. They are introduced into the mammalian cells by transfection with mammalian expression vectors (usually plasmids or retroviruses) encoding genes for these cellular marker proteins that have been modified with tags, such as green or red fluorescent proteins or epitope tags, which are recognized by antibodies conjugated to fluorescent dyes. The tags can be used to visualize the proteins inside the host cells by fluorescence microscopy. These are only a few examples of reagents and approaches that are currently being generated by cell biologists to study intracellular processes, but which are proving equally applicable to studies of bacterium-host cell interactions. Moreover, robotically controlled, high-throughput fluorescence microscopes are now available that allow rapid screening for changes in the infected host caused by bacterial mutants.

Organ Culture Models

Organs or portions of them can now be kept viable *in vitro* for longer and longer periods of time. Such tissues are called **organ cultures**. In the case of organ cultures, techniques for

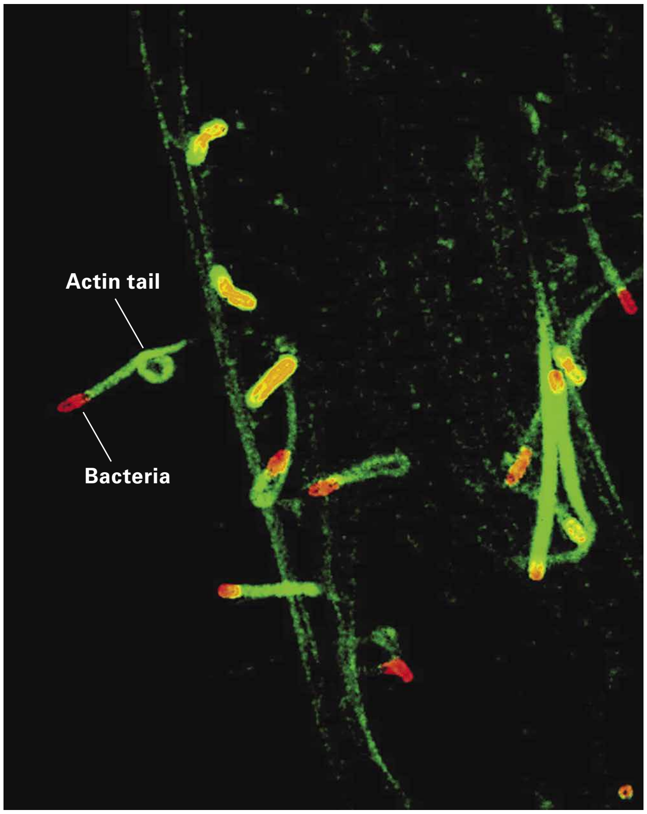

Figure 8-7. Visualization of intracellular bacteria and actin filaments. The intracellular pathogen *Listeria monocytogenes* stained with red fluorescent dye can be visualized by fluorescence microscopy using staining of actin with green fluorescent dye conjugated to phalloidin. Reprinted from Reyrat J-M, Telford JL. 1999. *Trends Microbiol* **7:**187–188, with permission.

in situ quantitative detection of proteins and mRNA make it possible to detect elevated expression of bacterial genes in a particular tissue. An advantage of using organ cultures is that there are usually multiple cell types present, including some of the immune system, which might allow a better approximation of what is happening during a natural infection. Organ cultures provide a much better model than tissue culture cells for what transpires in an animal or a human host, but they may be more difficult to obtain and maintain. An organ culture can begin to deteriorate within hours or days, making it difficult to do long-term experiments. Despite these challenges, there have been successes. One example is the organ culture model for the foodborne pathogen *C. jejuni*, in which researchers have used human gastrointestinal tissue obtained from endoscopic biopsies of patients to highlight the surprising propensity of *C. jejuni* to adhere to mucosal tissue via its flagellum rather than pili, which are more typically used by bacteria for surface adhesion. Such *ex vivo* organ culture models have been particularly helpful in cases like *C. jejuni*, where research has been hampered by lack of a suitable infection model.

Scientists who study the adherence of bacteria to skin cells have benefited enormously from the popularity of cosmetic surgery, which generates large amounts of skin tissue. Similarly, hysterectomies make fallopian tube and uterine tissues available. However, the availability of these tissues has dropped considerably in recent years due to a trend away from performing complete hysterectomies, and the number of people lining up to donate portions of their liver, heart, or brain has always been rather limited. Because of this limited supply of organ cultures from donors, scientists have begun to develop artificial organ cultures. One example of this is artificial skin equivalents, which are made by culturing a suspension of human foreskin fibroblasts in a matrix of native acid-soluble collagen and serum-supplemented medium at 37°C, where the collagen polymerizes and traps the cells, which then elongate and spread for several days. Then, a freshly isolated suspension of human skin-derived keratinocytes is seeded onto the surface of the collagen-fibroblast matrix, and the keratinocytes are allowed to grow to cover the surface, depositing basement membrane beneath them, differentiating into epidermal cells, and leading to formation of skin layers. Other connective-tissue, bone, heart, liver, and neuronal-tissue equivalents are also being developed, and these systems are beginning to be utilized for host-bacterium interaction studies. Indeed, some of the more exciting recent advances in the area of organ culture research have been in the development of **multiorgan microphysiological systems** that can serve as human surrogates for drug screening and testing. These microscale platforms enable organ-organ interactions in cell cocultures using microfluidic devices (Figure 8-8), where the medium connects the organ tissues through diffusion or circulation.

The Continuing Need for Reliable and Plentiful Information about Disease Pathology

A topic that is seldom discussed, but is very important for making progress in pathogenesis research, is the quality and quantity of information available about how a disease progresses and, perhaps even more important, the extent to which basic scientists in the field are aware of this information. There are some fields, like tuberculosis research, in which scientists are fortunate to have a vast store of information about how the disease progresses in the human body. Also, scientists working in the tuberculosis field are almost all conversant with this body of knowledge and appeal to it on a regular basis to assess whether their experimental findings make sense in the real-world context.

Unfortunately, this is not the case in all areas of pathogenesis research. To completely understand the complex nature of host-pathogen interaction and its disease outcome, it is extremely important for researchers to acquire

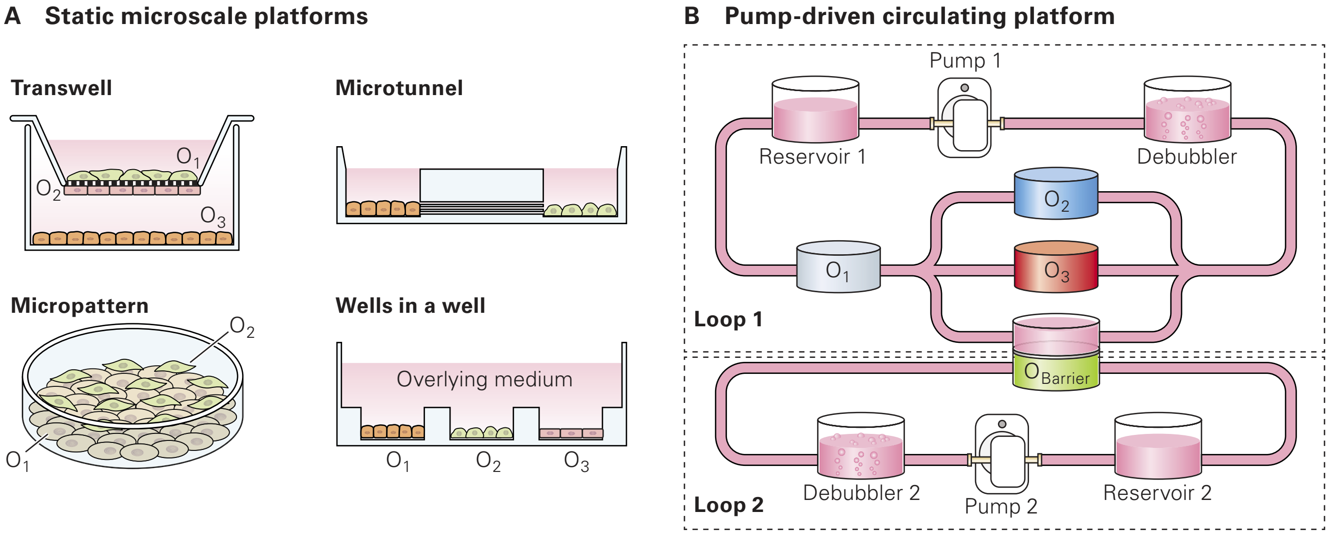

Figure 8-8. Multiorgan microphysiological systems. Shown are platforms for creating organ-organ interactions in cocultures. **(A)** In static multiorgan platforms, the connecting medium diffuses from one organ to the other in transwells, microtunnels, monolayer patterns, or large pores in wells. **(B)** In circulating multiorgan platforms, the connecting medium is pumped through the organ systems in serial or parallel, and sometimes with a porous membrane barrier between them. O_n, organ module. Adapted from Wang YI, Carmona C, Hickman JJ, Shuler ML. 2018. *Adv Healthc Mater* **7(2),** with permission.

key knowledge of human physiology or the physiology of whatever animal they are using as a model and to know as much as possible about the normal distribution of the bacteria in the body during infection, and about how and what type of damage occurs as the disease progresses. Against this background, when the physiological relevance is taken into consideration, the results of experiments on tissue culture cells and other ex vivo model systems are more likely to be interpreted correctly and to provide new insights that could lead to improved treatments.

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Questions

1. Discuss at least three ideal attributes of an animal model.

2. Using leeches as models for vampires is a humorous example of an animal model, yet it illustrates some of the problems encountered when choosing an animal model and then deciding how far to believe the results of animal model experiments. What problems and what advantages of animal models does this rather silly example illustrate?

3. What should the criteria be for a nonhuman animal model to be acceptable as a stand-in for humans? Under what conditions would you favor allowing human volunteers to be used as guinea pigs?

4. Describe the type of disease that would have a low ID_{50} but a high LD_{50}. Is it possible for there to be a disease for which LD_{50} is an appropriate measure but ID_{50} is not? Is there a combination of high/low ID_{50} and high/low LD_{50} that should not be possible?

5. When two bacterial strains are orally ingested, Bacterium A has an $ID_{50} = 10,000$ and Bacterium B has an $ID_{50} = 10$. But, when the bacteria are injected into a mouse model of infection, Bacterium A ($LD_{50} = 10$) is more virulent than Bacterium B ($LD_{50} = 10,000$). Explain how these results might be possible.

6. What limitations would organ cultures have as model systems?

7. Explain how it is possible to differentiate bacteria able to invade cultured epithelial cells from those only able to attach to the surface, and to determine the number of internalized bacteria (without using a microscope!).

8. Fill in the blank: The relative ability of a pathogen to cause disease of greater or lesser severity is that pathogen's _______.

9. Which of the following statements is true for LD_{50} and ID_{50} measurements regarding non-toxin mediated bacterial diseases?
   a. LD_{50} is a more accurate measure of virulence than ID_{50}.
   b. ID_{50} is a more accurate measure of virulence than LD_{50}.
   c. If the LD_{50} is low, then the ID_{50} is also low.
   d. If the LD_{50} is high, then the ID_{50} is always low.
   e. If the LD_{50} is high, then the ID_{50} is always high.

10. A researcher uses the gentamicin protection and plaque assays to identify virulence factors associated with an intracellular pathogen by comparing wild-type bacterium with five different mutants, each with a deletion in a gene encoding a putative virulence factor. The results are shown in the table.

Table 1.

BACTERIA	GENTAMICIN ASSAY		PLAQUE ASSAY PHENOTYPE OBSERVED
	NO. OF COLONIES WITHOUT GENTAMICIN	NO. OF COLONIES WITH GENTAMICIN	
Mut1	10^9	10^6	Large plaques
Mut2	10^9	10^5	No plaques
Mut3	10^9	10^2	Large plaques
Mut4	10^9	10^5	Small plaques
Mut5	10^2	1	No plaques
Wild type	10^9	10^6	Large plaques

Which of the following statements is/are correct?
   a. Mut1 is defective in a gene encoding a protein important for binding to the host cell.
   b. Mut2 is defective in a gene encoding a protein important for rapid cell-to-cell spread.
   c. Mut3 is defective in a gene encoding a protein necessary for survival in the phagolysosome.
   d. Mut4 is defective in a gene encoding a protein important for rapid cell-to-cell spread.
   e. Mut5 is defective in a gene encoding a protein necessary for binding to the host cell.

11. A researcher uses the gentamicin protection assay with lung epithelial cells to determine whether four different clinical isolates from the lungs of cystic fibrosis patients are intracellular bacterial pathogens. The results are shown in Figure 1 below.

    Which of the following statements is/are correct?

    a. Isolates #1, #2, and #3 are intracellular pathogens.
    b. Isolates #2, #3, and #4 are extracellular pathogens.
    c. Isolate #1 is an intracellular pathogen.
    d. Isolates #1, #3, and #4 bind lung cells.
    e. Isolates #2, #3, and #4 bind lung cells.
    f. Isolate #2 does not bind lung cells.
    g. Isolate #2 is an intracellular pathogen.

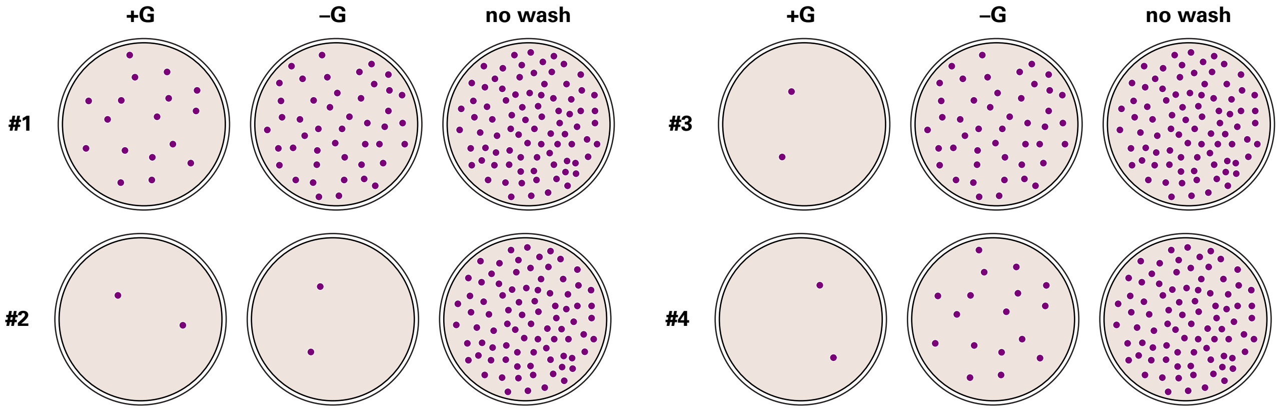

Figure 1. **Results of gentamicin protection assay.**

Solving Problems in Bacterial Pathogenesis

1. A group of researchers at the USDA isolate a new bacterium from lungs and lymph nodes of several young horses that became ill and died at a local stable. Prior to death, symptoms included disorientation and loss of motor function, so the researchers suspected central nervous system involvement, which was confirmed by the observation of brain lesions in the dead animals. Based on 16S rRNA comparison, the bacterium is distantly related to *Neisseria meningitidis*, and they subsequently named it *Neisseria equiniae*.

    a. The researchers developed a horse model of infection. During their studies, they isolated two avirulent mutants (NeMut1 and NeMut2) that were still able to grow as well as wild-type *N. equiniae in vitro*, but not *in vivo*. They also found that these mutants had deletions in genes encoding putative virulence factors. To confirm that the disrupted genes in the two mutants indeed encoded proteins important for virulence, the researchers compared the mutants to wild-type (wt) bacterium in the horse model of infection. The results are summarized in the following Table 1.

Table 1.

STRAIN	DOSE (CFU)	MORTALITY ON DAY 3 (NO. DEAD/ TOTAL NO.)	NO. (CFU) OF BACTERIA RECOVERED FROM LYMPH NODES ON DAY 3
Wt	10	2/10	10^3
Wt	10^2	3/10	10^5
Wt	10^3	6/10	10^8
Wt	10^4	9/10	10^{11}
Wt	10^5	10/10	10^{12}
NeMut1	10^5	0/10	10
NeMut1	10^7	0/10	10^3
NeMut1	10^9	0/10	10^5
NeMut2	10^5	0/10	10^3
NeMut2	10^7	2/10	10^5
NeMut2	10^9	3/10	10^8

    Determine the LD_{50} and ID_{50} values for each of the wild-type and mutant strains. Interpret your results.

    b. The researchers decided to determine the competitive index (CI) of each of the mutants, again using the horse infection model. The results are summarized in Table 2.

Table 2.

STRAINS USED	NO. OF BACTERIA IN INOCULUM (CFU MUTANT:CFU WT)	NO. OF BACTERIA RECOVERED FROM LYMPH NODES ON DAY 3 (CFU MUTANT:CFU WT)
NeMut1:wt	$10^5:10^5$	$10:10^{12}$
NeMut2:wt	$10^5:10^5$	$10^2:10^9$
NeMut1:NeMut2	$10^5:10^5$	$10^3:10^6$

Determine the CI for NeMut1:wt, NeMut2:wt, and NeMut1:NeMut2. Interpret your results. Compare the results obtained from the LD_{50} and ID_{50} values with those obtained from the CI studies.

2. A group of researchers have isolated a new Bacterium W from the lymph nodes of several patients that returned from a camping trip and presented in the emergency room with high fever, rash, and swollen lymph nodes. Based on 16S rRNA sequence comparison, they determined that this new bacterium is distantly related to the Gram-negative bacterium *Francisella tularensis*. The researchers developed a tissue culture model of invasion using a phagocytic cell line and a mouse model of infection (via injection). They found that Bacterium W binds to and invades phagocytic cells. Using a mouse model of infection, the researchers isolated two avirulent mutants of W that they call Wmut1 and Wmut2, both of which grow as well as wild type *in vitro*. In the tissue culture model for invasion, Wmut2 can still bind to and invade the phagocytic cells just as well as wild-type W, while Wmut1 cannot.
    a. To confirm that the genes disrupted in the two mutants indeed encoded proteins important for virulence, the researchers compared the mutants to wild-type (wt) Bacterium W in the mouse model of infection. The results are summarized in Table 3.

Table 3.

W STRAIN	DOSE (CFU)	MORTALITY ON DAY 3 (NO. DEAD/ TOTAL NO.)	MORTALITY ON DAY 7 (NO. DEAD/ TOTAL NO.)	NO. (CFU) OF BACTERIA RECOVERED FROM LYMPH NODES ON DAY 3
Wt	10	2/10	10/10	10^3
Wt	10^2	3/10	10/10	10^5
Wt	10^3	6/10	10/10	10^8
Wt	10^4	9/10	10/10	10^{11}
Wt	10^5	10/10	10/10	10^{12}
Wmut1	10^5	0/10	0/10	10
Wmut1	10^7	0/10	0/10	10^3
Wmut1	10^9	0/10	0/10	10^5
Wmut2	10^5	0/10	0/10	10^3
Wmut2	10^7	0/10	1/10	10^5
Wmut2	10^9	1/10	2/10	10^8

Determine the LD_{50} and ID_{50} values for each of the wild-type and mutant strains. Interpret your results.

    b. The researchers decided to determine the competitive index (CI) of each of the mutants, again using the mouse model. The results are summarized in Table 4.

Table 4.

W STRAINS USED	NO. OF BACTERIA IN INOCULUM (CFU MUTANT:CFU WT)	NO. OF BACTERIA RECOVERED FROM LYMPH NODES ON DAY 3 (CFU MUTANT:CFU WT)
Wmut1:wt	$10^5:10^5$	$10:10^{12}$
Wmut2:wt	$10^5:10^5$	$10^3:10^9$
Wmut1:Wmut2	$10^5:10^5$	$10^3:10^8$

Determine the CI for Wmut1:wt, Wmut2:wt, and Wmut1:Wmut2. Interpret your results. Compare the results obtained from the LD_{50} and ID_{50} values with those obtained from the CI studies.

3. Your research laboratory has been working for several years on a new bacterium that was isolated from the lungs of two emergency room patients that became ill several days after a vacation trip to a popular resort hotel called Paradise. According to 16S rRNA comparison, this bacterium is related to the Gram-negative *Klebsiella pneumoniae*, and you named this new bacterium *Klebsiella paradisiae*. Your laboratory developed a tissue culture model of invasion using bovine lung cell line and a rabbit lung model of infection, and during their studies, your students isolated five avirulent mutants (Mut1 through Mut5) in the rabbit lung model of infection, which were deleted in genes encoding putative virulence factors. Your students conducted a series of experiments to determine the role of each of these virulence factors in pathogenesis. Based on the results shown in the table below, predict the possible function(s) of each of the putative virulence factors deleted in Mut1 through Mut5 and provide at least one experiment that you could perform for each that could be used to confirm your prediction.

Table 5.

BACTERIA	GENTAMICIN ASSAY		PLAQUE ASSAY PHENOTYPE OBSERVED
	NO. OF COLONIES WITHOUT GENTAMICIN	NO. OF COLONIES WITH GENTAMICIN	
Mut1	10^9	10^6	Large plaques
Mut2	10^9	10^5	No plaques
Mut3	10^9	10^2	Large plaques
Mut4	10^9	10^5	Small plaques
Mut5	10^2	1	No plaques
Wild type	10^9	10^6	Large plaques

9

IN THIS CHAPTER

- **Finding a Needle in a Haystack**
- **Biochemical Approaches**
  - Isolation and Purification of Toxic Factors
- **Molecular Genetic Approaches**
  - Screening Using Recombinant Genes
  - Reporter Fusions
  - Mutagenesis Screening
- **Genome-wide Sequencing Approaches for Identifying Virulence Genes**
  - Tn-Seq Technology to Identify *In Vivo*-Expressed Genes
  - RNA-Seq Technology to Identify *In Vivo*-Expressed Genes
  - Comparative Genomic Sequence Analysis for Identifying Virulence Genes
- **Proteomics Approaches for Identifying Virulence Factors**
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CHAPTER 9

Identification of Virulence Factors

MOLECULAR APPROACHES FOR BACTERIAL FACTORS

Imagine that you are a doctor, and a patient enters your clinic. What do you do to figure out what is ailing that patient? First, you would probably ask the patient a series of questions about how they feel and what happened to them to determine whether they received an injury or are suffering from a disease. You would probably ask them about their personal or familial history to identify potential genetic factors. Then, you might order blood tests or an X ray or an MRI (magnetic resonance imaging), depending on the patient's responses, to further narrow down what could be affecting the patient. Finally, from all of this information, you might formulate a diagnosis to explain the patient's symptoms and suggest a course of treatment. Now, imagine that you are a microbiologist and instead of a patient, you have a bacterial pathogen that is able to cause a disease. What do you do to figure out what makes that bacterium a pathogen? It turns out, the process for identifying virulence mechanisms is not all that different from a doctor diagnosing a patient. First, you might ask what the nature of the disease is that the bacterium causes. Next, you might ask what kind of genetic factors the bacterium has that might contribute to the bacterium's ability to survive in the host and cause the disease symptoms. Then, you could perform various tests to close in on a set of potential virulence factors. Ultimately, the main difference between identifying these virulence factors and diagnosing a patient is that unlike with patients, you can't just ask the pathogen a bunch of questions. Instead, microbiologists use biochemical, genetic, and bioinformatic tools to indirectly interrogate pathogens and their role in disease.

Finding a Needle in a Haystack

Thirty years ago, recombinant DNA technology started a line of research that has enabled the discovery and characterization of a wide array of genes associated with virulence in pathogenic bacteria. It was no longer necessary to painstakingly isolate and purify a bacterial component to demonstrate that it was toxic or caused certain disease symptoms to verify that it was a virulence factor. Advances in molecular genetic technologies allowed for more rapid discovery of virulence genes and along with it the discovery of virulence traits previously not suspected.

The field of bacterial pathogenesis is once again being transformed, this time through next generation high-throughput and whole genome sequencing technologies, combined with automation and powerful new bioinformatics tools. These technologies have revolutionized the way we approach the identification of virulence factors. The new data provided by complete knowledge of a bacterium's genetic makeup and the differential patterns of gene and protein expression, revealed through current multi-omics technologies, dwarfs previous methods of virulence gene discovery. Indeed, the vast and exhaustive list of new candidates revealed by comparative genomic analysis of virulent and avirulent strains of the same bacterium or between closely related species is overwhelming. The challenges now for researchers are to characterize these candidates, to annotate the gene functions, and to determine their roles in pathogenesis.

However, just because we have this new technology, does this make all of the previous approaches obsolete? Recent experience says not. It is becoming increasingly clear that a combination of new multi-omics approaches with older genetic and classical biochemical techniques is highly effective at identifying and gaining a better understanding of the functions of virulence factors. Those who become trapped in a single new technology generally fall by the wayside scientifically, so it is important to understand a variety of old and new techniques. Not only are many of the older methods still useful, but they are constantly being updated and improved to keep them relevant and useful. Most interesting of all, a recent trend has been a return to the consideration of bacterial physiology, the reason being that genetic and biochemical analyses alone do not make the final step in understanding how all this information plays out in the living cell.

Biochemical Approaches

Isolation and Purification of Toxic Factors

The first virulence factors to be identified and characterized at the molecular level were bacterial protein toxins produced by some pathogens to elicit harmful effects on eukaryotic host cells and cause disease. These toxins could be purified from culture filtrates of the pathogenic bacteria that produce them by using various biochemical approaches based on their intrinsic biochemical properties, including filtration, centrifugation, selective precipitation, and chromatography (anionic, cationic, hydrophobic, size exclusion, or affinity) (Figure 9-1). As part of satisfying Koch's postulates, the purified toxic factor can then be reintroduced into a susceptible animal or exposed to sensitive host cells to reproduce some or all of the disease symptoms associated with the original infection (for more on the actions of these toxins see chapter 12). Two important historical examples of bacterial toxins of note are diphtheria toxin (produced by *Corynebacterium diphtheriae*) and cholera toxin (produced by *Vibrio cholerae*).

The discovery of diphtheria toxin in culture medium from *C. diphtheriae* by Friedrich Loeffler (1852–1915) in 1884 heralded a new era in medical microbiology. Shortly thereafter in 1888, Émile Roux (1853–1933) and Alexandre Yersin (1863–1943) demonstrated that cell-free bacterial culture filtrates produced characteristic death of internal organs upon injection into experimental animals and could kill sensitive animal cells in tissue culture. Between 1890 and 1894, Emil von Behring (1854–1917) developed an **antitoxin** from serum of horses immunized against *C. diphtheriae* and showed that this horse-derived antitoxin, which contained toxin-neutralizing antibodies, could be successfully used to treat diphtheria, for which he received the Nobel Prize in 1901. The bacteriologists Anna Wessels Williams (1863–1954) and William H. Park (1863–1939) discovered methods to produce large quantities of diphtheria antitoxin and thereby greatly increase its availability in combating this devastating disease. For a famous account of the use of diphtheria antitoxin that saved a town in 1925, see Box 12-1. It was not until the 1930s that vaccination with heat- or chemically inactivated toxin (diphtheria toxoid) with alum salts as an adjuvant was widely used and shown to afford complete protection from subsequent exposure to the bacteria, and it was not until the 1960s that its mechanism of action could finally begin to be understood at the molecular level.

The connection of cholera disease to water supplies contaminated with a microbe was made as early as 1854 by John Snow (see Figure 6-8), but it was not until 1883 that Robert Koch identified *V. cholerae* as the disease-causing microbe, although he too had some doubts at first (see Box 6-1). Part of this doubt came from the finding that not everyone exposed to *V. cholerae* through ingestion developed cholera diarrhea and that there is no animal model for cholera disease. Interestingly, purified cholera toxin at first did not appear to have any obvious effects on cultured mammalian cells, and even intramuscular injection with an active toxin did not seem to provide adaptive immune protection from cholera diarrhea. This observation misled many

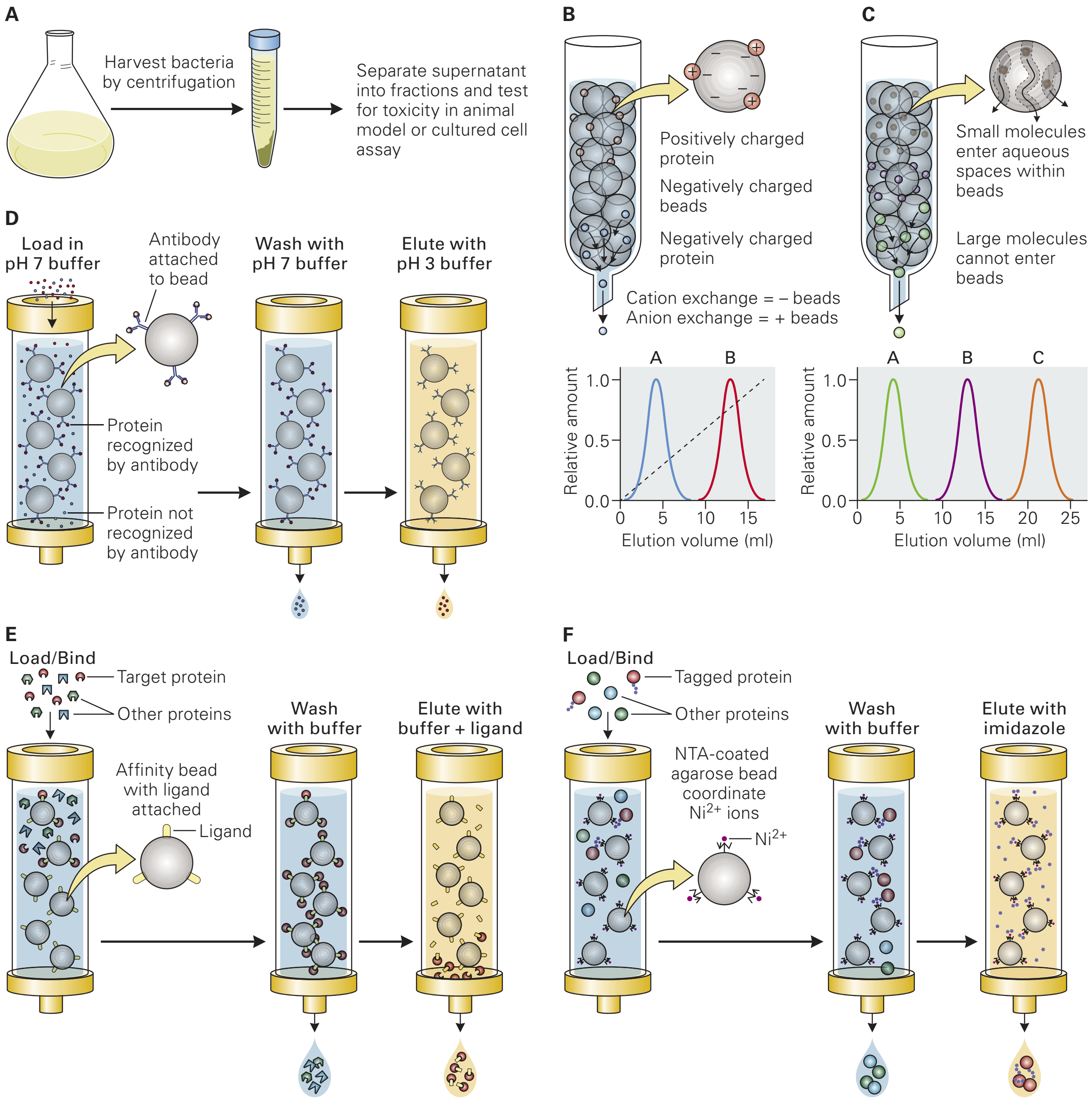
A
Harvest bacteria by centrifugation
Separate supernatant into fractions and test for toxicity in animal model or cultured cell assay
B
Positively charged protein
Negatively charged beads
Negatively charged protein
Cation exchange = – beads
Anion exchange = + beads
C
Small molecules enter aqueous spaces within beads
Large molecules cannot enter beads
A
B
1.0
0.5
0.0
Relative amount
0
5
10
15
Elution volume (ml)
A
B
C
1.0
0.5
0.0
Relative amount
0
5
10
15
20
25
Elution volume (ml)
D
Load in pH 7 buffer
Antibody attached to bead
Wash with pH 7 buffer
Elute with pH 3 buffer
Protein recognized by antibody
Protein not recognized by antibody
E
Load/Bind
Target protein
Other proteins
Affinity bead with ligand attached
Ligand
Wash with buffer
Elute with buffer + ligand
F
Load/Bind
Tagged protein
Other proteins
NTA-coated agarose bead coordinate Ni2+ ions
Ni2+
Wash with buffer
Elute with imidazole

Figure 9-1. Identifying virulence factors by purification from bacterial culture supernatants. (A) The first step in the isolation of a potential virulence factor from bacterial cultures involves centrifugation to pellet the bacterial cells and filtration of the cell-free supernatant, which is further processed to separate the toxic factor from other cellular components, usually through one or more chromatography steps. The separate fractions are then tested for toxicity in animal models or cultured mammalian cell assays. **(B)** Separation of proteins based on intrinsic charge through ion exchange chromatography. In a buffer with a pH that is below a protein's isoelectric point (pI), the protein will have a positive charge at pH7. In cation exchange chromatography shown here, a protein B with a more positive charge (a pI >7) attaches to the negatively charged beads of the column, while a more negatively charged protein A will not adhere as well to the column beads and so will elute first from the column. The positively charged protein B can then be eluted from the column by increasing the salt concentration of the elution buffer (denoted by dashed line). The salt cations compete with the positive charges of the protein B for binding to the negatively charged beads. In anion exchange chromatography, the beads are positively charged and will bind to the more negatively charged protein A, while the more positively charged protein B will elute first. **(C)** Separation of proteins based on molecular size through size exclusion or gel filtration chromatography. The beads of the column contain aqueous pores or spaces, into which smaller proteins such as C can enter, thereby slowing down their progress through the matrix. Larger proteins, such as A, are excluded from some or all of the bead spaces, and so they elute more quickly, while medium-sized proteins such as B are partially retained. **(D)** Separation of proteins based on affinity chromatography using specific affinity tags. This separation method takes advantage of the highly specific interaction between an antibody and its antigen (immunoaffinity chromatography). Cell lysates are mixed or poured into a column with the affinity beads, the beads are washed to remove unbound components, and then the affinity-tagged protein is eluted from the column with low pH. Eluted proteins are then assayed for activity. **(E)** Separation of proteins based on affinity chromatography using specific ligands. This separation method takes advantage of highly specific interactions that some proteins have with certain molecules called ligands. These ligands are covalently attached to a bead resin. Cell lysates are then mixed with or poured into a column with the bead resin. The beads are then washed to remove unbound components. The affinity-tagged protein is eluted from the column by addition of buffer containing the ligand, which competes with the bead-bound ligand for binding to the protein. **(F)** Separation of proteins based on metal-chelation chromatography. This separation method uses the tight-binding affinity of a recombinant protein of interest that is engineered to have a hexahistidine (His_6)-affinity tag and its cognate ligand, in this case a Ni^{2+}-NTA ligand attached to a bead (Ni^{2+}-chelation chromatography). Cell lysates are mixed or poured into a column with the affinity beads, the beads are washed to remove unbound components, and then the affinity-tagged protein is eluted from the column by addition of imidazole, which is a competing ligand that binds Ni^{2+} instead of the His_6-tag. Eluted proteins are then assayed for activity.

researchers to believe that cholera toxin was not the virulence factor present in *V. cholerae* culture filtrates that was responsible for the profuse diarrhea. As it turns out, cholera toxin changes the cellular signaling processes of the intestinal cells (see chapter 12), yet does not damage or kill them. Furthermore, the diarrheal effect of cholera toxin is very specific for the type of cells present in the intestine, so intradermal or intramuscular injection with cholera toxin naturally does not produce many symptoms. Instead, intradermal or intramuscular injection with cholera toxin leads to the development of humoral (IgG antibody) immune response, which is less effective than a mucosal (sIgA antibody) immune response against a gut pathogen, such as *V. cholerae*. It was not until much later, when genetic approaches were developed that reduced virulence in a mutant strain of *V. cholerae* with the cholera toxin gene deleted, and when a better diarrheal model system using a rabbit ileal loop assay (as described in chapter 2) was developed, that cholera toxin could finally be demonstrated to be the etiological agent of cholera diarrhea.

Like these two historical examples, many other bacterial protein toxins were first identified after being isolated from bacterial cultures. Other proteins (proteases, nucleases, glycosidases, and lipases) that are secreted or released into the culture medium can be isolated and characterized in a similar way by using biochemical separation methods. Once a protein toxin has been purified, its amino acid sequence can be determined by using mass spectrometry methods, which enables the identification of the toxin gene in the sequenced genome of the bacterium that produces it. Once the gene is identified, it can be cloned into and expressed from a separate plasmid in a laboratory strain of a bacterium, such as *E. coli*, with prior approval by an institutional biosafety committee. Nowadays, unique properties are often introduced into these cloned proteins (also called recombinant proteins) to facilitate purification. For example, an affinity tag is a protein sequence motif that binds to specific chemical structures that can be attached to beads (see Figure 9-1D). This approach allows for rapid, large-scale production of the purified protein. Highly specific proteases are often used to cleave off affinity tags before the purified proteins are characterized biochemically and for activities related to virulence.

Unlike the above examples, many virulence factors are not proteins. Cell surface glycolipids or carbohydrates can confer on the bacteria the ability to adhere to human tissues and/or the ability to trigger uptake of a bacterium by a normally nonphagocytic cell. Other types of secreted molecules, besides proteins, have been shown by biochemical approaches to contribute to disease. In these cases, the composition and structure of the carbohydrates, lipids, and other secondary metabolites (small molecular-weight compounds, such as antibiotics, quorum-sensing molecules, or toxins) can be determined by using nuclear magnetic resonance (NMR), mass spectrometry, X ray crystallography, and other analytical methods. The structure of these molecules can, in turn, give hints about the genes that mediate their biosynthesis. For example, strains of the opportunistic pathogen *Pseudomonas aeruginosa* are frequently isolated from the lungs of individuals with cystic fibrosis and the wounds of burn patients. *P. aeruginosa* produces a variety of redox-active phenazine-derived compounds, all of which have antibiotic properties, but also cause toxicity in the host (Figure 9-2A). These natural products were first isolated in the late 19th century as secondary metabolites using biochemical methods, including extraction with organic solvents (e.g., chloroform) and purification by crystallization. Current biochemical separation and characterization methods also include high-pressure liquid chromatography (HPLC) and analysis by UV spectroscopy and mass spectrometry (Figure 9-2B).

Biochemical approaches were also used to identify a particularly elusive and novel virulence factor from *Mycobacterium ulcerans,* an emerging human pathogen harbored by aquatic insects and the causative agent of Buruli ulcers, a devastating disease that has emerged as a serious health threat in Australia and western and central Africa. Infection with *M. ulcerans* causes progressive necrotic lesions that, if untreated, can extend over 15% of a victim's body and lead to lifelong disability and occasionally death. Surprisingly, there is little inflammation or pain associated with the large skin lesions even in advanced stages of this disease. When first observed in the late 1960s and early 1970s, it was believed that the extensive necrosis was due to a toxin that diffuses beyond the infection site where the extracellular bacteria are colonized. Early attempts to define the biochemical properties of this toxin from cell-free culture filtrates suggested that it was a heat-stable substance, but efforts to isolate the toxin proved unsuccessful. The nature of the toxin thus remained unknown until 1998, when a group of investigators at Rocky Mountain Laboratories used organic solvents to extract and eventually purify the lipid-soluble toxins from bacterial culture filtrates. Structural analyses using NMR and mass spectrometry determined that the biologically active substances were a mixture of lipid-like polyketide-derived macrolides, which they called mycolactones (see Figure 12-3). The fact that these mycolactones appeared to be polyketide-derived compounds suggested that it might be possible to identify the biosynthetic genes based on their similarity to other polyketide biosynthetic genes. This approach enabled researchers to subsequently discover a large 174-kb plasmid in *M. ulcerans* that was absent in closely related strains of *Mycobacterium marinum,* but which contained two gene clusters encoding two large polyketide synthases plus polyketide modifying enzymes responsible for mycolactone production.

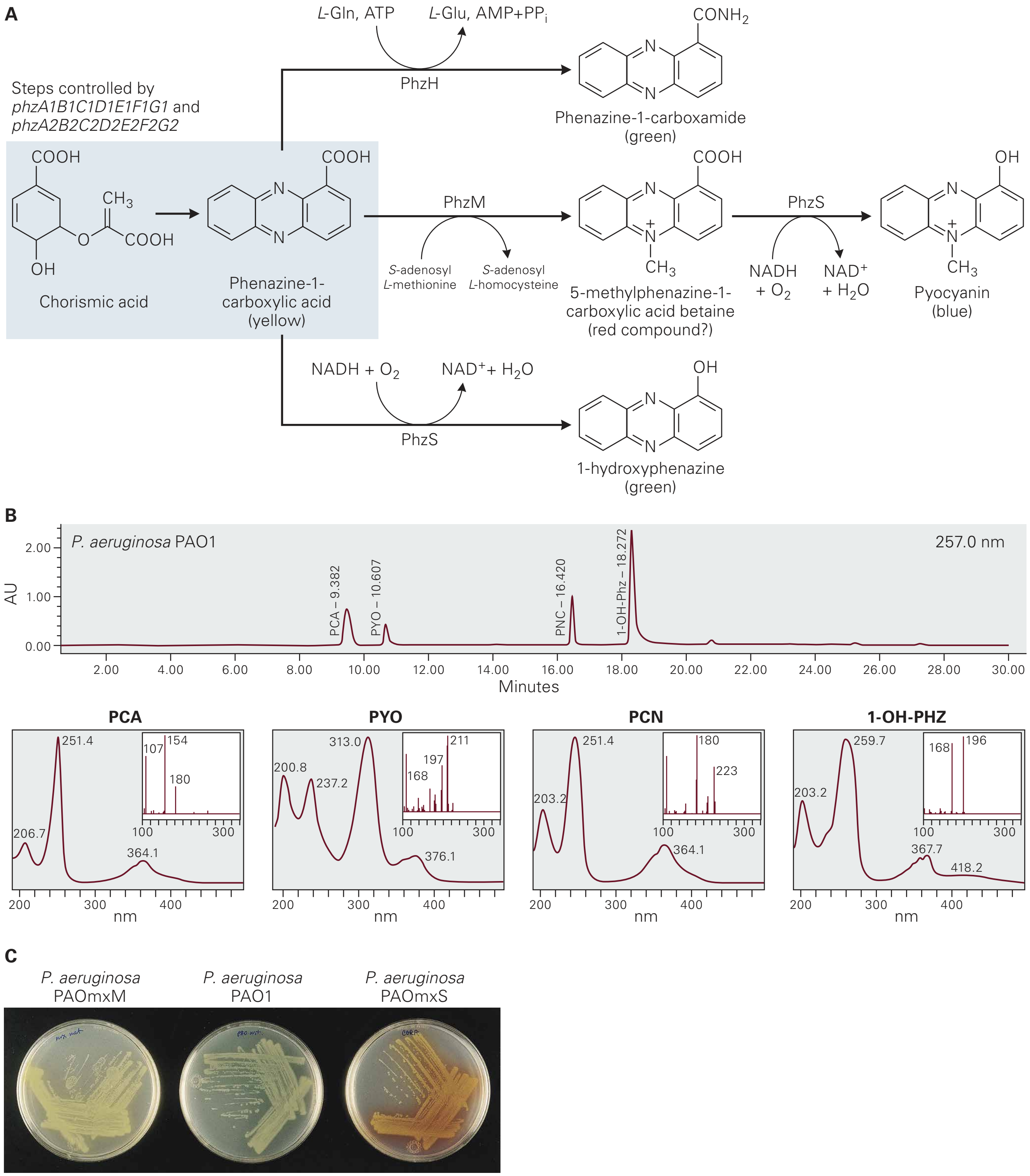
A
L-Gln, ATP
L-Glu, AMP+PP$_i$
PhzH
CONH$_2$
Phenazine-1-carboxamide (green)
Steps controlled by phzA1B1C1D1E1F1G1 and phzA2B2C2D2E2F2G2
COOH
CH$_3$
O
COOH
OH
Chorismic acid
COOH
Phenazine-1-carboxylic acid (yellow)
PhzM
S-adenosyl L-methionine
S-adenosyl L-homocysteine
COOH
CH$_3$
5-methylphenazine-1-carboxylic acid betaine (red compound?)
PhzS
NADH + O$_2$
NAD$^+$ + H$_2$O
OH
CH$_3$
Pyocyanin (blue)
NADH + O$_2$
NAD$^+$+ H$_2$O
PhzS
OH
1-hydroxyphenazine (green)
B
P. aeruginosa PAO1
257.0 nm
AU
2.00
1.00
0.00
PCA – 9.382
PYO – 10.607
PNC – 16.420
1-OH-Phz – 18.272
2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00 26.00 28.00 30.00
Minutes
PCA
251.4
107
154
180
206.7
364.1
100 300
200 300 400
nm
PYO
313.0
211
197
168
200.8
237.2
376.1
100 300
200 300 400
nm
PCN
251.4
180
223
203.2
364.1
100 300
200 300 400
nm
1-OH-PHZ
259.7
168
196
203.2
367.7
418.2
100 300
200 300 400
nm
C
P. aeruginosa PAOmxM
P. aeruginosa PAO1
P. aeruginosa PAOmxS

Figure 9-2. Phenazine-derived toxins produced by *Pseudomonas aeruginosa*. Strains of the opportunistic pathogen *Pseudomonas aeruginosa* produce a variety of redox-active phenazine-derived compounds, all of which have antibiotic properties, but also cause toxicity in the host. These natural products were first isolated in the late 19th century as secondary metabolites using biochemical methods, including extraction with organic solvents (e.g., chloroform) and purification by crystallization. Current biochemical methods also include separation and analysis by high-performance liquid chromatography (HPLC), UV spectroscopy, and mass spectrometry. **(A)** For example, the strain *P. aeruginosa* PAO1 produces the blue pyocyanin (PYO), green phenazine-1-carboxamide (PCN), yellow phenazine-1-carboxylic acid (PCA), and green 1-hydroxyphenazine (1-OH-PHZ). Shown is the proposed biosynthetic pathway for production of phenazines in *P. aeruginosa* PAO1. In PAO1, two 7-gene operons are responsible for production of the intermediate PCA precursor, which is then converted to the PYO, PCN, and 1-OH-PHZ by additional modifying enzymes, PhzH, PhzM, or PhzS. **(B)** Biochemical characterization of chloroform extracts containing phenazine compounds produced by *P. aeruginosa* PAO1 by HPLC analysis (top, chromatographs of elution profile with detection at 257 nM), UV spectroscopy, and mass spectrometry (bottom, UV spectra for each compound with corresponding mass spectra as insets). **(C)** Visualization of production of colored phenazine compounds: Colonies of wild-type *P. aeruginosa* PAO1 (center plate) are blue due to production of PYO. Colonies of the mutant strain PAOmxM (left plate) with a deletion in the gene *phzM* were yellow due to accumulation of PCA and loss of PYO production. Colonies of the mutant strain PAOmxS (right plate) with a deletion in the gene *phzS* did not produce PYO, but accumulated a dark red water-soluble pigment, presumably the intermediate metabolite 5-methylphenazine-1carboxylic acid. Adapted from Mavrodi DV, Bonsall RF, Delaney SM, Soule MJ, Phillips G, Thomashow LS. 2001. *J Bacteriol* **183:**6454–6465, with permission.

Molecular Genetic Approaches

Screening Using Recombinant Genes

One effective classical approach to identifying the traits that contribute to virulence is to clone the genes from the pathogen of interest into an avirulent strain of a bacterium, such as *E. coli*, and screen for mutants that are now virulent. An example might be to clone the toxin gene from a pathogenic strain into a nontoxigenic, avirulent strain of *E. coli* and demonstrate that the resulting strain produces the recombinant protein toxin and is now virulent. In another example, ordinary laboratory strains of *E. coli* do not adhere to or invade cultured epithelial cells, but *Salmonella enterica* does. Selecting for *E. coli* clones that contain *Salmonella* genomic DNA segments and screening for *E. coli* clones that adhere to and/or invade the tissue culture cells enabled the identification of potential *Salmonella* adhesins and/or invasins (Figure 9-3A). A similar approach confirmed that the *hlyA* gene from the Gram-positive pathogen *Listeria monocytogenes* encodes the hemolysin listeriolysin O (LLO), which allows for bacterial escape from an endosomal vacuole into the cytosol. In this case, the introduction of the *hlyA* gene into a laboratory strain of *Bacillus subtilis* facilitated its escape from the normal phagolysosomal degradation pathway and growth in the host cell cytosol (Figure 9-3B).

Cloning strategies have worked in some cases to identify candidate virulence genes, but they have some important limitations. Because standard cloning procedures isolate only relatively small portions of the total bacterial genome (fewer than 30 kilobase pairs), this approach works best if only one or a few contiguous genes from the pathogen are sufficient to give the desired phenotype. Also, this approach requires that the gene(s) of interest be expressed in *E. coli* (or whatever avirulent bacterium is being used). Not surprisingly, this approach has been most successful when applied to bacterial species that are closely related to *E. coli* or another avirulent bacterium.

Reporter Fusions

You will learn more about the regulation of virulence genes later in this book, but since identification of virulence genes often requires the exploitation of their differential regulation under selective conditions (e.g., off outside the host and on when in the host), we will introduce here a few basic concepts to assist with understanding how reporter systems work in identifying virulence genes. Until very recently researchers often used **reporter genes** to identify virulence genes based on their up or down regulation by a common promoter-activator system. Nowadays, these reporter gene-based screening approaches are often used to confirm the differential regulation of the putative virulence genes and to further characterize the nature of their regulation.

Experimentally, identification of potential virulence genes by their regulatory properties is done using **reporter fusions** (also called **operon fusions**). When the virulence gene has already been cloned, a **transcriptional** or **translational fusion** can be constructed (Figure 9-4). In either case, the reporter fusion links the regulatory circuit of a virulence gene (*vir*) that we want to study to a **reporter gene**, which is usually an enzyme whose activity is easy to measure or detect (e.g., antibiotic resistance, production of a colored pigment, luminescence, etc.) or a protein with specific measurable properties (e.g., fluorescence). Table 9-1 lists some of the most commonly used reporter genes.

In the example of a transcriptional fusion shown in Figure 9-4A, the ribosome-binding site (RBS) and the ATG-start site of the hybrid construct are provided by the reporter gene *lacZ*. Although RNA polymerase starts transcription from the regulated promoter region of the virulence gene (P/O *vir*), the β-galactosidase protein is translated from the segment of the mRNA transcript beginning at the AUG site that corresponds to the start of the *lacZ* gene. Thus, β-galactosidase expression occurs only under conditions when the virulence gene is transcribed, and differences in β-galactosidase specific activity can occur in bacteria grown under different conditions. For example, if the original virulence gene is transcribed at higher levels in bacteria at higher temperature levels (e.g., at 37°C compared to 25°C), β-galactosidase specific activity in the bacteria expressing the fusion reporter gene will be greater at 37°C compared to 25°C.

The β-galactosidase activity used in the above examples is readily detected on plates or in liquid assay systems by using **chromogenic substrates**. A popular chromogenic substrate is **indolyl-galactoside (X-gal)**, which turns blue when its galactosyl bond is cleaved by the enzyme. In the example mentioned above, colonies containing a fusion of *lacZ* to the temperature-regulated virulence gene would be blue at 37°C and white or light blue at 25°C. Not only is it easier to assay β-galactosidase activity than it is to assay most virulence proteins, but β-galactosidase poses none of the hazards associated with, for example, assaying a toxin. The type of medium and growth conditions used to differentiate whether a putative virulence factor gene is expressed *in vivo*, but not *in vitro*, during growth on laboratory medium can be problematic. For example, Lactose-MacConkey agar has been used to differentiate Lac$^+$ from Lac$^-$ colonies. This medium contains bile salts, which may be an important signal *in vivo* for gastrointestinal pathogens. Thus, β-galactosidase fusions to certain virulence genes will appear to be expressed *in vitro* because of the presence of bile salts in this indicator medium. In another example, body temperature is an inducer of some important virulence genes. However, this regulation will be missed if bacteria containing β-galactosidase fusions to certain virulence genes are only examined on indicator plates incubated at 37°C, instead of

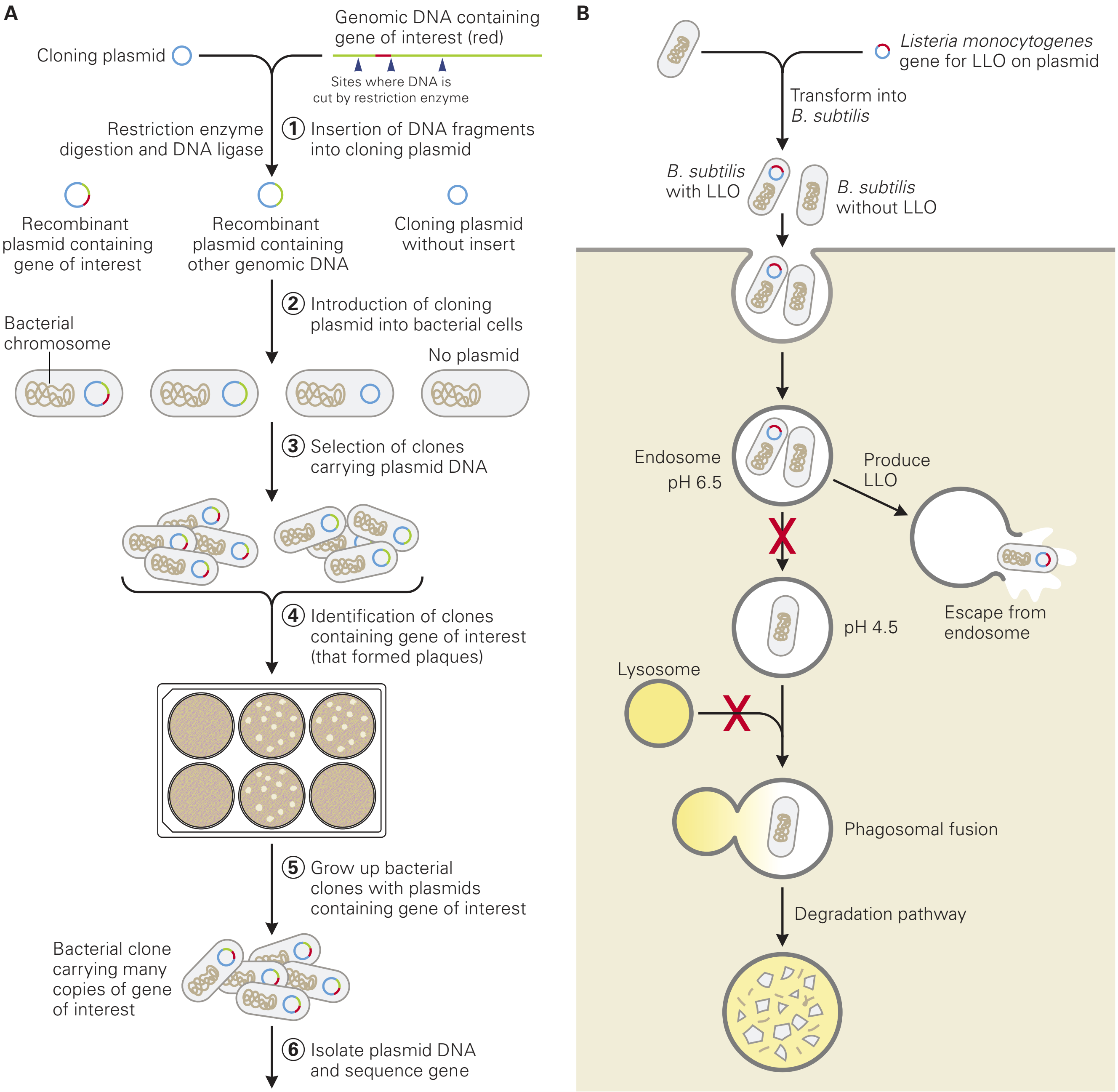
A
Cloning plasmid
Genomic DNA containing gene of interest (red)
Sites where DNA is cut by restriction enzyme
Restriction enzyme digestion and DNA ligase
① Insertion of DNA fragments into cloning plasmid
Recombinant plasmid containing gene of interest
Recombinant plasmid containing other genomic DNA
Cloning plasmid without insert
② Introduction of cloning plasmid into bacterial cells
Bacterial chromosome
No plasmid
③ Selection of clones carrying plasmid DNA
④ Identification of clones containing gene of interest (that formed plaques)
⑤ Grow up bacterial clones with plasmids containing gene of interest
Bacterial clone carrying many copies of gene of interest
⑥ Isolate plasmid DNA and sequence gene
B
Listeria monocytogenes gene for LLO on plasmid
Transform into B. subtilis
B. subtilis with LLO
B. subtilis without LLO
Endosome pH 6.5
Produce LLO
Escape from endosome
pH 4.5
Lysosome
Phagosomal fusion
Degradation pathway

Figure 9-3. Identifying virulence genes by cloning and expressing them in avirulent bacterial strains. (A) Example for Gram-negative bacteria: Cloning fragments of genomic DNA from an invasive *Salmonella* strain into a laboratory strain of *E. coli* enabled the isolation of clones of *E. coli* that could invade tissue culture cells. Sequencing of the cloned fragments helped to identify *Salmonella* invasion genes. **(B)** Example for Gram-positive bacteria: Cloning the gene for the hemolysin listeriolysin O (LLO) from the opportunistic pathogen *Listeria monocytogenes* into a laboratory strain of *Bacillus subtilis* allowed the *B. subtilis* to escape from the host phagosome and grow in the cytoplasm.

at 25°C and 37°C. These examples show that careful attention must be given to the medium and growth conditions used to determine whether putative virulence genes are expressed *in vitro* as well as *in vivo*.

In the case of a translational fusion (Figure 9-4B), the RBS and ATG-start site of the hybrid construct are provided by the virulence gene (*vir*) and all or part of the *vir* gene is fused with the reporter gene, here *phoA*, which does not have its own RBS/ATG site. Again, expression of alkaline phosphatase (PhoA) reporter enzymes occurs under the same conditions as the virulence gene, but now the reporter is covalently attached to the end of the virulence protein, Vir-PhoA. Because the recombinant fusion protein must be expressed as a functional enzyme for activity to be observed, it is necessary that the two genes, *vir* and *phoA*, are joined together with their protein-coding sequences in-frame. Translational fusions of virulence genes with reporters can provide valuable information about the subcellular location and secretion properties of the expressed virulence genes. For example, Vir-PhoA fusions are usually employed for studying membrane-associated or secreted proteins (Figure 9-4C). PhoA is inactive in the cytoplasm of the bacteria and must be secreted onto the surface and into the periplasm or medium in order to fold properly and exhibit alkaline phosphatase activity. Translational fusion reporters are also used to study post-transcriptional regulation of virulence genes (discussed further in chapter 14).

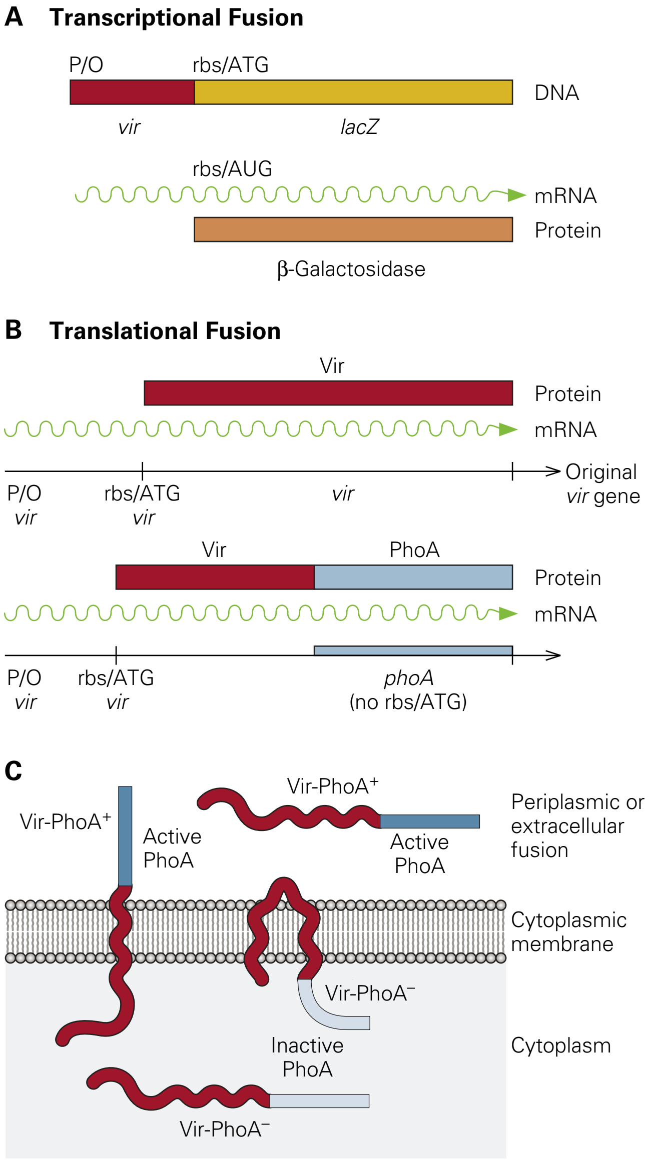

Figure 9-4. Organization of a reporter gene system. (A) For a transcriptional reporter gene system, a DNA segment containing a virulence gene promoter/operator (P/O *vir*) is fused to a promoterless reporter gene, such as that encoding β-galactosidase (*lacZ*). The mRNA transcript of the fusion construct is initiated from the promoter of the virulence gene (*vir*), whereas the ribosome binding site (RBS) located just upstream from the start codon (ATG/AUG) and the protein reading frame encoding β-galactosidase are from the *lacZ* gene. Thus, β-galactosidase expression from the fusion construct reflects transcriptional initiation and regulation from the *vir* P/O promoter. **(B)** For a translational reporter gene system, a DNA segment containing the virulence gene promoter/operator (P/O *vir*) and the *vir* gene RBS and AUG start codon are fused in-frame to the reading frame of the reporter gene, such as that encoding alkaline phosphatase (*phoA*). The mRNA of the fusion construct initiates from the P/O *vir* promoter and encodes a fusion of the virulence protein or a truncation thereof with the reporter protein (i.e., Vir-PhoA) that is translated from the RBS/AUG start codon of the virulence gene. Thus, in this case, β-galactosidase expression from the fusion construct represents transcription and translation control of the *vir* gene. **(C)** Identification of genes encoding virulence factors that are secreted or expressed on the surface of a bacterium can be identified by making translational fusions of the *vir* gene with *phoA*. PhoA is a periplasmic protein that is inactive in the cytoplasm of bacteria, but becomes active when it is secreted onto the surface in the periplasm or medium. Since the N terminus of the virulence protein (Vir) contains the sequence information that defines where the fusion protein will be located in the bacterium, PhoA activity can be used to determine whether the virulence protein is cytoplasmic, membrane associated, or secreted.

Mutagenesis Screening

Ever since the discovery that genes are encoded by DNA, scientists have been using mutagenesis to link specific DNA sequence elements to measurable phenotypes. The basic approach has largely remained unchanged for decades: (1) observe a phenotype in an organism, (2) mutagenize the organism, (3) isolate mutants that no longer have that phenotype, and (4) figure out what DNA elements in the mutant were changed. When applied toward identification of virulence factors, the phenotype of interest is the ability to produce toxic compounds or more generally the ability to colonize and/or cause disease. Individual mutants can then be individually tested for virulence or observed competing against each other inside a host to determine the relative infectivity of the different mutants. Alternatively, separate mutants can be screened for their ability to produce toxic compounds associated with disease. For example, we mentioned above that *P. aeruginosa* produces various toxic phenazine-based compounds. Mutagenesis screens were instrumental to the identification of the biosynthetic pathways for several of these compounds (see Figure 9-2C). Once the mutated or disrupted gene has been identified, it can be cloned and further characterized by expression and purification of the recombinant protein in an appropriate laboratory bacterial strain, followed by confirmation of its capacity to confer the expected virulence

Table 9-1. **Commonly used reporter gene systems**

REPORTER GENE	REPORTER ENZYME	VISUALIZATION ASSAY
lacZ	β-Galactosidase (β-Gal)	This is a colorimetric assay in which cells that express β-Gal appear blue when grown on medium containing a substrate analog, such as X-Gal. X-Gal is cleaved by β-Gal into galactose and 5-bromo-4-chloro-3-hydroxyindole, which is then oxidized to 5,5′-dibromo-d,d′-dichloro-indigo, an insoluble blue product. For spectrophotometric analysis, the substrate *o*-nitrophenyl-β-D-galactoside (ONPG) is often used. There are also fluorescent dye-labeled substrates for fluorescence microscopic analysis.
uidA	*E. coli* β-glucuronidase (GUS)	The most common substrate for GUS histochemical staining is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc); the product of the reaction is blue. Other common substrates are *p*-nitrophenyl- β-D-glucuronide for the spectrophotometric assay and 4-methylumbelliferyl-beta-D-glucuronide (MUG) for the fluorometric assay.
cat	CAT	CAT covalently attaches an acetyl group from acetyl-coenzyme A to the antibiotic chloramphenicol. The conversion of a less hydrophobic substrate to a more hydrophobic product can be detected chromatographically.
phoA	PhoA	PhoA is a hydrolase enzyme responsible for removing phosphate groups from various substrates, such as the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP or BCIP), the product of which turns dark blue in the presence of an oxidant, such as nitroblue tetrazolium chloride (NBT). Bacterial PhoA is a periplasmic protein, so it is often used as a reporter for exported secreted proteins.
gfp	GFP	GFP is a protein from the jellyfish *Aequorea Victoria* that fluoresces green when exposed to blue light. Cells that express GFP glow green under UV fluorescent light. Genetic mutants of GFP fluoresce at different emission maxima: yellow fluorescent protein (YFP, Citrine, Venus), blue fluorescent protein (EBFP2), and cyan fluorescent protein (CFP, mTurquoise, Cerulean).
rfp	RFP	RFP is a tetrameric red fluorescent protein, originally isolated from the sea anemone *Discosoma* and denoted as DsRed. Genetic engineering has generated improved variants of RFP that fold more rapidly and are monomeric (mScarlet), as well as others that fluoresce with different emission maxima: blue (mTagBFP), orange (mOrange), bright red (mCherry), or far-red (mRasberry).
luc	Luc	The enzyme luciferase converts luciferin substrate to produce oxyluciferin and energy in the form of light (chemiluminescence).
luxCDABE	*Photorhabdus luminescens* luciferase (Lux)	A bacterial luciferase reporter that is also used extensively in bacterial systems. The operon mediates synthesis of the bacterial luciferase substrate, so the substrate does not need to be added.
bla *erm* *tet* *kan* *aadA* *cat*	β-lactam resistance (β-lactamase) Erythromycin resistance Tetracycline resistance Kanamycin resistance Spectinomycin resistance Chloramphenicol resistance	Antibiotic resistance genes can be used for selection screening on agar plates containing the antibiotic.

properties to the avirulent strains in an appropriate infection model.

When generating mutants, different mutagens can be used, ranging from chemical mutagens (such as ethyl methanesulfonate [EMS] or ethidium bromide) to UV irradiation to transposons. Each has their own strengths and weaknesses in terms of ease of use, bias in where in the genome mutations occur, frequency of mutations in a single genome, and difficulty in identification of the mutated genes. Chemical mutagenesis and UV irradiation are generally less biased in terms of what genes can be targeted. However, while it is relatively easy to generate the mutants and show attenuation or identify which mutants are avirulent, until recently, it has often been nontrivial and labor-intensive to find the actual genes that have been altered in the mutant bacteria because these mutagenesis methods do not provide an easily selectable marker or other indication of where the mutation has occurred. This problem has now been largely obviated by the availability of low-cost, highly accurate methods to sequence whole genomes of bacteria and thereby to locate mutations (see below and chapter 5). Even spontaneous mutations that arise in the absence of mutagenesis can now be rapidly identified by whole-genome sequencing.

Transposon insertion mutagenesis is another widely used strategy for identifying virulence genes. Transposons were introduced in chapter 7. Transposon insertion mutations are generated by nearly random insertion of a transposon into the genome of a pathogen, and the resulting insertion mutants are screened for loss of virulence (Figure 9-5). Although some transposons are somewhat biased in terms of the sequences they target, it is now much easier to identify which genes have been disrupted compared to older genetic mapping methods. The transposons commonly used in current approaches carry a selectable marker (e.g., an antibiotic resistance gene), or a reporter gene, such as *lacZ* encoding β-galactosidase or *lux*-encoding luciferase. By introducing a transposon into the bacterial genome and selecting for colonies expressing the selectable marker, the investigator generates a collection or library of mutants, where each mutant contains a single transposon insertion and many of these mutations disrupt a gene. This has been an important approach for bacterial species lacking sophisticated genetic mapping tools needed to locate and clone point mutations caused by single base-pair changes or by small deletions.

A few limitations of the transposon mutagenesis approach should be kept in mind. First, transposons can carry transcriptional terminators. If a transposon lands in the first gene in an operon, it will eliminate expression of not only that gene but also downstream genes (i.e., transposon insertions are often polar). The avirulent phenotype of a mutant could thus be due to loss of expression of the gene interrupted by the transposon or due to loss of expression of downstream genes in the operon. Alternatively, some antibiotic resistance genes in transposons contain promoters but no terminators, and aberrant increased expression from genes downstream of transposon insertion sites can cause phenotypes. Thus, it is necessary to confirm the observed results. One approach is to construct additional mutants with in-frame deletions of each of the genes in the operon containing the gene that was disrupted by transposon insertion. A more common approach is to perform a standard genetic complementation test, where a wild-type copy of the transposon-disrupted gene is reintroduced at a second, ectopic site (e.g., on a plasmid or at a genetically neutral site on the chromosome) in the mutant bacterium to restore gene expression and thereby distinguish polar effects on downstream genes in operons. It should be noted that repairing the transposon-disrupted gene back to a wild-type copy (i.e., wild-type gene knock-in or repair) does not eliminate polarity as a possible explanation for phenotypes of the original transposon insertion mutation.

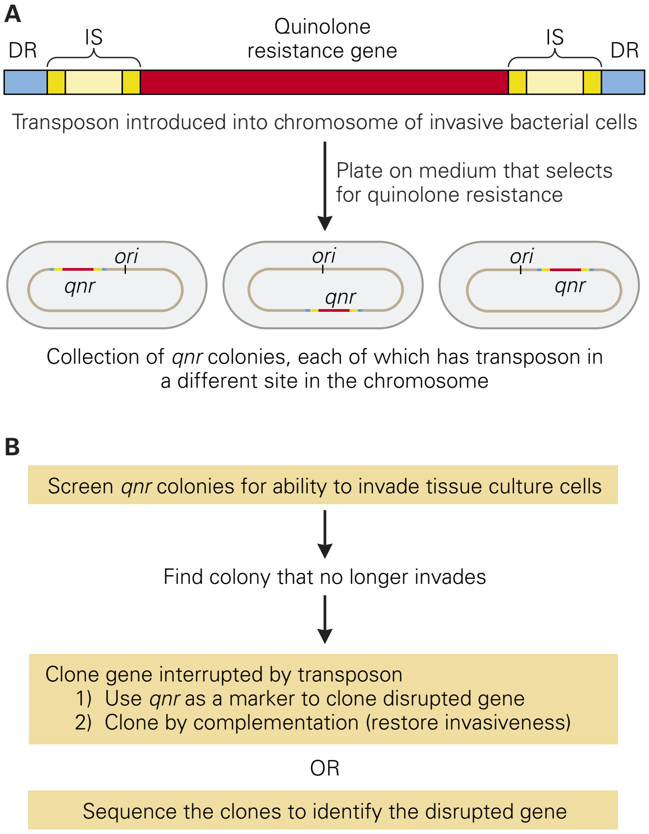
A
DR
IS
Quinolone resistance gene
IS
DR
Transposon introduced into chromosome of invasive bacterial cells
Plate on medium that selects for quinolone resistance
ori
qnr
ori
qnr
ori
qnr
Collection of qnr colonies, each of which has transposon in a different site in the chromosome
B
Screen qnr colonies for ability to invade tissue culture cells
Find colony that no longer invades
Clone gene interrupted by transposon
1) Use qnr as a marker to clone disrupted gene
2) Clone by complementation (restore invasiveness)
OR
Sequence the clones to identify the disrupted gene

Figure 9-5. Identifying virulence genes by traditional transposon insertion mutagenesis. Transposons carrying selectable markers, such as antibiotic resistance genes, can be used to identify genes that are important for survival in the environment and in the host (i.e., virulence genes). **(A)** For example, a transposon with a quinolone resistance gene (*qnr*) as a selection marker can be used to identify virulence genes for an invasive intracellular bacterium. A library of transposon insertion mutants can be screened for those that no longer are able to invade and survive in the host. **(B)** Traditional methods for identifying genes needed for invasion of tissue culture cells by an invasive intracellular bacterium include cloning of the gene disrupted by the transposon and testing for virulence or reintroduction of the gene on an expression plasmid for complementation of the mutation. Nowadays, the chromosomes of the clones are directly sequenced to identify the disrupted gene.

A second limitation of transposon mutagenesis is that disruptive insertion mutations can only be obtained in genes that are not essential for growth on a selective medium *in vitro*. A transposon insertion into a gene that is essential for growth, such as the genes encoding ribosomal proteins or subunits of RNA polymerase, will not be isolated because the bacteria will not survive to form a colony. This might be considered an advantage if one assumes that the most interesting virulence genes are the ones that are not expressed by bacteria growing in a laboratory medium, but rather are genes that are induced specifically under conditions that mimic those found in an animal host. On the other hand, the lack of growth of a mutant *in vitro* may be dependent on the medium or condition which could confuse interpretation about whether the disrupted gene is a virulence factor. Moreover, a broader view of what constitutes a virulence factor is discussed at the end of this chapter.

A third disadvantage of transposon mutagenesis, or any mutagenesis approach for that matter, is the occurrence of separate bacterial genes with redundant functions. Sometimes two homologues of a gene are present in a bacterial chromosome, such that knocking out one of the copies does not result in a phenotype. Both copies of redundant genes need to be knocked out at the same time to see a strong phenotype, which is usually a synthetically lethal phenotype. Adaptations of the Tn-Seq method described later in this chapter can be used to identify functionally redundant genes whose absence results in a synthetically lethal phenotype.

Often investigators use transposons carrying a promoterless *lacZ* gene in addition to a selectable antibiotic resistance marker to locate genes that respond to specific conditions or signals (Figure 9-6). Selection for antibiotic resistance generates a set of random insertions of the transposon into the bacterial chromosome. Colonies carrying transposon insertions are then screened for regulated expression of *lacZ*. Only a fraction of the transposon insertions will fuse *lacZ* to promoters and only a fraction of these fusions will be regulated in the desired manner. For example, suppose the genes being sought are expressed at a high level only under low iron conditions. As illustrated in Figure 9-6A, colonies carrying transposon insertions would be replica plated onto a high iron medium and a low iron medium, both of which contain X-gal. Colonies containing the desired fusions would be blue on the low-iron plates but white on the high-iron plates.

To find regulatory genes that control expression of putative virulence genes, mutant strains are made with the promoter region of the putative virulence genes fused with reporter genes, such as the *lacZ* reporter (Figure 9-6B). The strain is then mutagenized using another transposon, and the resulting colonies are screened either for those exhibiting aberrant regulation (i.e., colonies that do not turn blue under inducing conditions, indicating a possible loss of an activator) or colonies that turn blue under both inducing and noninducing conditions (indicating a possible loss of a repressor).

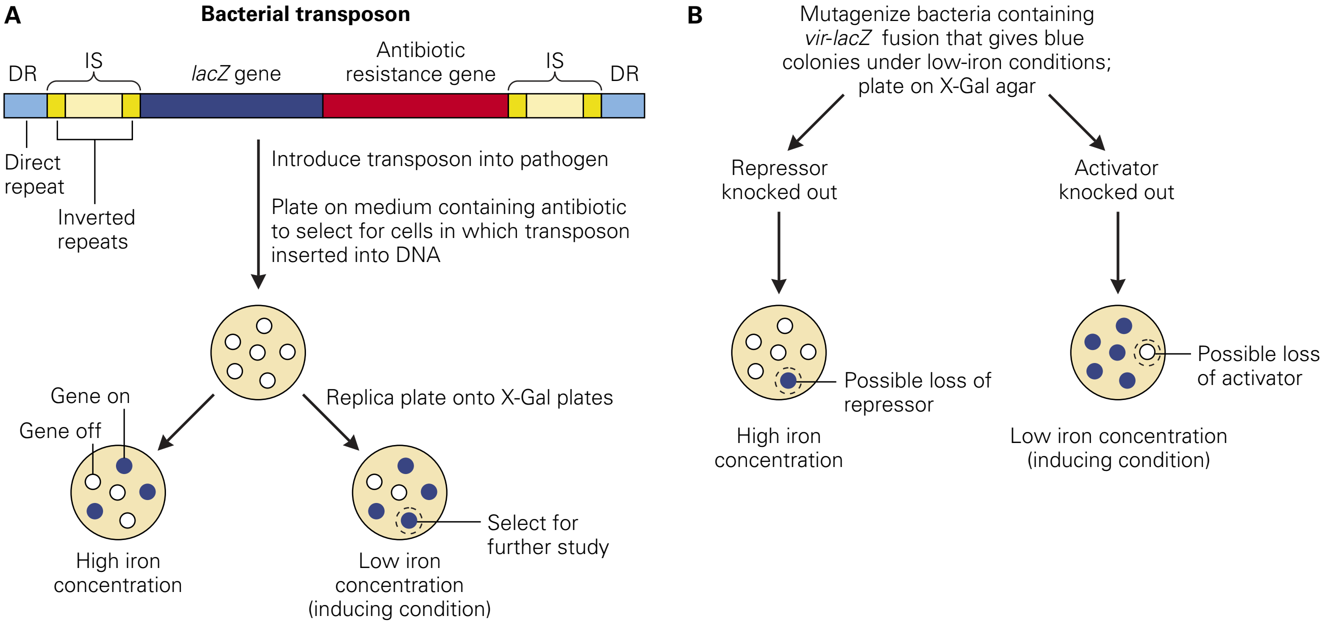
A
Bacterial transposon
DR
IS
lacZ gene
Antibiotic resistance gene
IS
DR
Direct repeat
Inverted repeats
Introduce transposon into pathogen
Plate on medium containing antibiotic to select for cells in which transposon inserted into DNA
Gene on
Gene off
Replica plate onto X-Gal plates
Select for further study
High iron concentration
Low iron concentration (inducing condition)
B
Mutagenize bacteria containing vir-lacZ fusion that gives blue colonies under low-iron conditions; plate on X-Gal agar
Repressor knocked out
Activator knocked out
Possible loss of repressor
Possible loss of activator
High iron concentration
Low iron concentration (inducing condition)

Figure 9-6. Using traditional transposon insertion mutagenesis approaches to identify genes regulated under different conditions. (A) Transposons carrying selectable transcriptional reporters, such as *lacZ*, can be used to identify genes that are regulated under different conditions, such as the presence of high or low iron concentrations. Transposase allows the transposon carrying the promoterless *lacZ* to integrate randomly into the genome. The selectable marker gene for antibiotic resistance allows for selection of colonies that have the transposon. A colony containing a *lacZ* fusion to a gene that is turned on under low iron conditions is screened on X-gal plates and chosen for further characterization. IS, insertion sequence in transposon. **(B)** Transposon mutagenesis can be used to find genes responsible for regulating virulence genes under different conditions. In this case, transposons carrying a second gene for selectable antibiotic resistance (without a *lacZ* reading frame) insert nearly randomly into the chromosome of a bacterium containing a reporter system, such as the one carrying a *vir-lacZ* fusion that is induced by low iron described above. The transposon mutagenized bacteria carrying the second transposon are replica plated onto X-gal plates containing either high or low iron concentrations. Mutants that no longer show differences in expression of β-galactosidase under the two conditions are likely to have transposons inserted into a regulatory protein: An activator if the colonies are white under both conditions; or a repressor if the colonies are blue under both conditions.

Once a gene has been tagged by a *lacZ* fusion or by a transposon insertion, it can readily be identified by sequencing. Importantly, current advances in Illumina and PacBio sequencing technologies (see chapter 5) now enable low-cost, high-coverage whole genome sequencing to identify sites of mutations or insertions rapidly. Other low-cost, pocket-sized, portable sequencing platforms, such as **nanopore sequencing technology**, are starting to appear for use in the field and classroom. For example, the MinION sequencer from Oxford Nanopore Technologies can carry out hundreds of kilobase reads in real-time that are recorded on a laptop computer (Figure 9-7). This technology currently can be used to identify microbes and to generate draft scaffold genome sequences of bacterial and viral genomes. However, the accuracy of reading a sequencing does not yet match that of the Illumina technology, which can be used reliably to identify single base-pair changes in whole bacterial genomes. With the availability of these increasingly easy-to-use, inexpensive sequencing tools, researchers are moving back to chemical or UV irradiation-generated mutagenesis methods or isolating spontaneous mutations, particularly for organisms that lack suitable transposon mutagenesis systems.

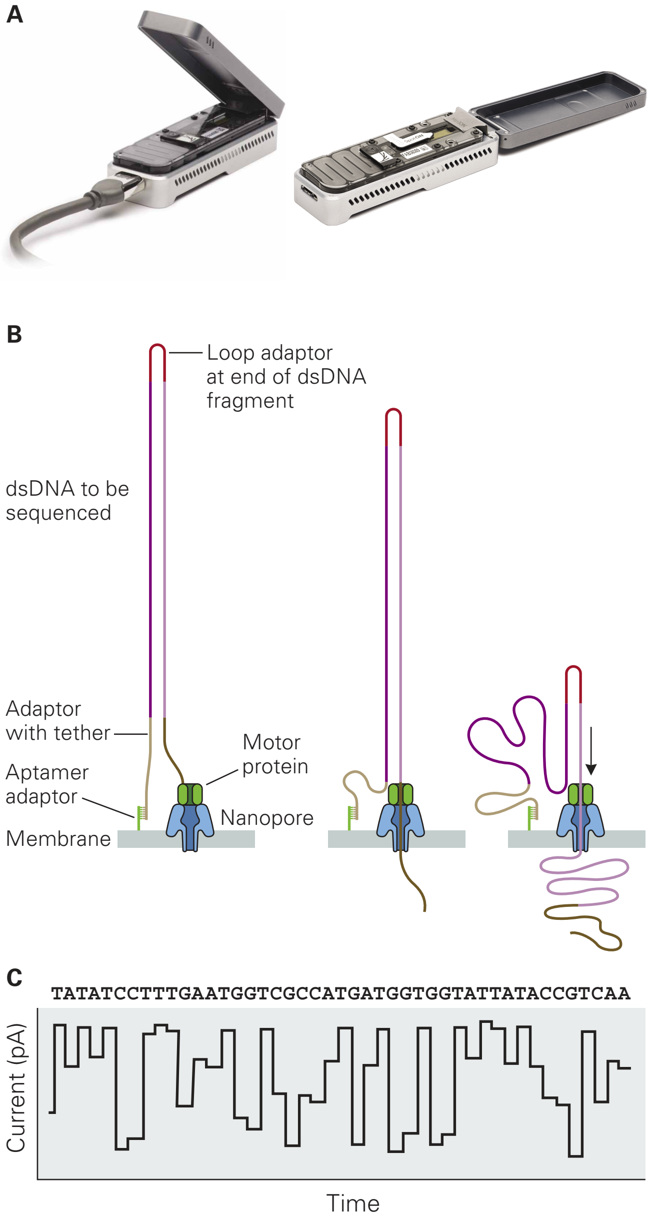

Figure 9-7. Nanopore sequencing technology using the MinION platform. (A) The MinION portable sequencer from Oxford Nanopore Technologies is a small, pocket-sized flow-cell device that can be plugged into a laptop computer that streams data in real time. Image courtesy of Nanopore (https://nanoporetech.com/products/minion). **(B)** A biological nanopore inserted into an electrically resistant synthetic membrane comprises the core of the MinION sequencer platform. Each flow cell is comprised of an array of microscaffolds, with each microscaffold supporting a membrane with a single embedded nanopore. The library genomic dsDNA fragment to be sequenced contains a hairpin loop adaptor at one end and is tethered to the membrane using an aptamer adaptor attached to a strand at the other end, while the complementary lead strand is bound to a motor protein. The motor protein with the bound DNA docks with the nanopore and facilitates passage of the lead strand followed by the rest of the DNA through the pore. Adapted from Jain M, Olsen HE, Paten B, Akeson M. 2016. *Genome Biol* **17:**239, with permission. **(C)** An electric potential is applied across the membrane, resulting in ion flow. Each microscaffold has its own electrode that is connected to a channel in the sensor array chip. Multiple nanopore experiments are conducted in parallel, depending on the number of channels. DNA bases in the nanopore disrupt the ion current in a manner that is dependent on their nucleotide sequence. Adapted from Leggett RM, Clark MD. 2017. *J Exp Bot* **68:**5419–5429, with permission.

Genome-wide Sequencing Approaches for Identifying Virulence Genes

Tn-Seq Technology to Identify *In Vivo*-Expressed Genes

Tn-Seq technology is an extremely powerful new approach for discovering *in vivo*-expressed virulence genes, particularly to study the requirement of specific genes for bacterial survival in certain environmental niches, such as in the lung versus the nasopharynx or in the intestine or the blood. Tn-Seq combines transposon mutagenesis with *in vivo* selection using an animal model of infection to screen for mutants that do not grow in the host (Figure 9-8). The mutants containing transposons are pooled and grown *in vitro*. The resulting culture is then split. The first part, called the input pool, is used to identify the relative ratio of mutants growing in culture *in vitro*, and the other part is used to inoculate an animal. After sufficient time for the infection to develop, an infected organ or blood is harvested, and the bacteria growing in it are recovered. The relative ratio of mutants in this output pool is then determined. DNA is extracted from the input

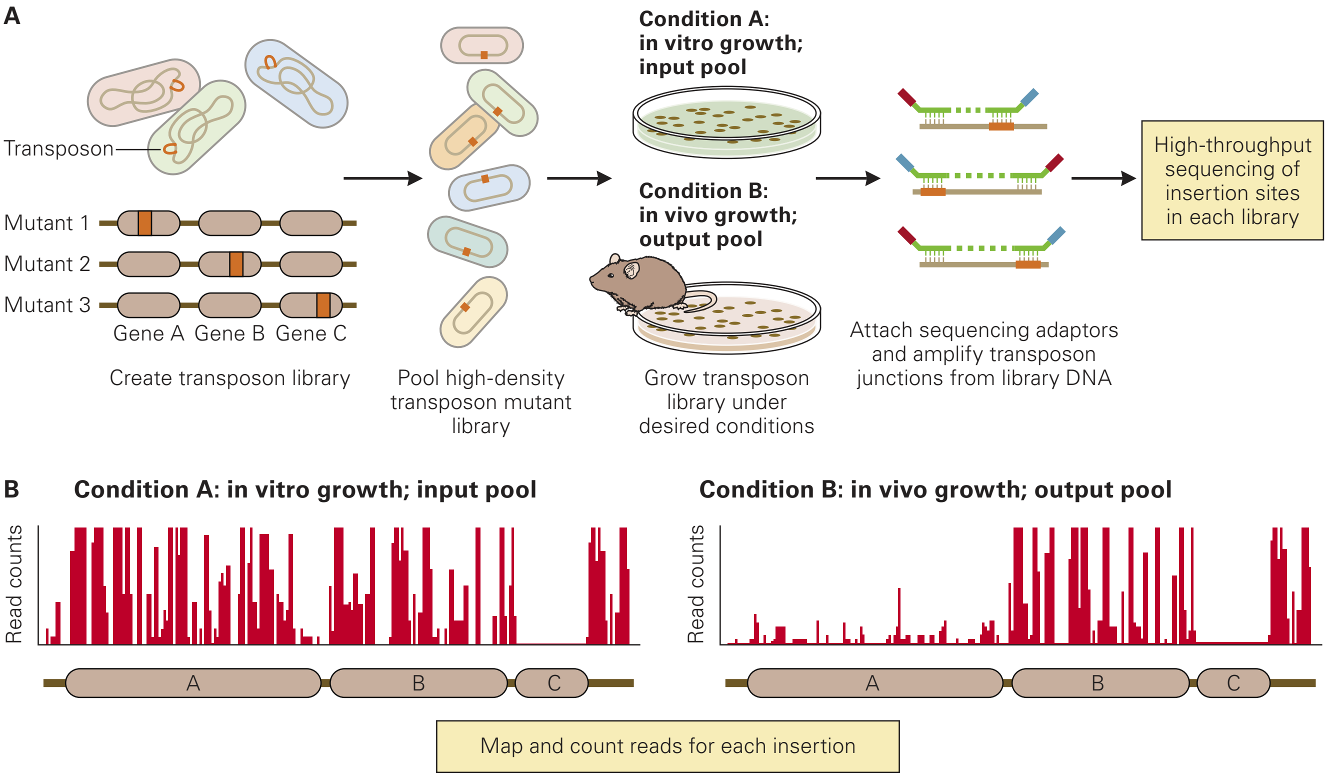
A
Transposon
Mutant 1
Mutant 2
Mutant 3
Gene A Gene B Gene C
Create transposon library
Pool high-density transposon mutant library
Condition A: in vitro growth; input pool
Condition B: in vivo growth; output pool
Grow transposon library under desired conditions
Attach sequencing adaptors and amplify transposon junctions from library DNA
High-throughput sequencing of insertion sites in each library
B
Condition A: in vitro growth; input pool
Condition B: in vivo growth; output pool
Read counts
Read counts
A
B
C
A
B
C
Map and count reads for each insertion

Figure 9-8. Transposon-Sequencing (Tn-Seq) Technology. In transposon-directed insertion-site mutagenesis and sequencing (Tn-Seq), transposon insertion mutagenesis is combined with massively parallel next-generation Illumina sequencing to identify genes that contribute to bacterial survival under different conditions. **(A)** A library of transposon insertion mutants is generated, and following initial plating on antibiotic plates, colonies containing separate insertions are pooled and stored. The pooled library of transposon mutants is then grown under two different conditions, such as in nonselective rich culture medium (input pool; condition A) versus in a host or animal model of colonization or infection (output pool; condition B). The bacterial DNA from the input and output pools is isolated, and the junctions between the transposons and the adjoining chromosomal DNA are amplified and sequenced. **(B)** Comparison of the read counts obtained from Illumina sequencing of the transposons inserted into each gene in both the bacteria in the input and output conditions enables the identification of genes essential for growth under both conditions (gene C in example), not required for growth in either condition (gene B in example), or survival *in vivo* (gene A in example). Gene A would be considered as encoding a putative virulence factor, which would be further analyzed by other methods described in this chapter. Adapted from Chao MC, Abel S, Davis BM, Waldor MK. 2016. *Nat Rev Microbiol* **14:**119–128, with permission.

(*in vitro* growth) and output (animal growth) pools of bacteria, and subjected to Illumina or nanopore sequencing.

By specifically initiating all sequencing reads from the edge of the inserted transposons to the adjacent bacterial chromosome, one can determine the precise location of where each transposon landed and which nonessential gene was disrupted. Multiple transposon insertions are detected in each nonessential gene in the input sample of bacteria grown in nonselective rich medium *in vitro*. By comparing which genes have transposon insertions in the input (*in vitro*) pool but lack transposon insertions in the output (*in vivo*) pool, it is possible to identify which genes cannot tolerate disruption when the bacteria are grown *in vivo*. These genes are presumably required for host colonization or survival inside the host during infection. These putative virulence genes can be involved in a number of different processes ranging from *in vivo* nutrient acquisition to gene regulation to adherence to toxin production. Additional experimentation is then done to verify the role of each of these putative virulence genes in the colonization and infection processes.

An advantage of Tn-Seq is that it is a negative selection method for identifying putative virulence genes based on mutations in genes that do not allow for survival in the host animal. Mutant bacteria can be constructed with deletions in these putative virulence factors for rapid comparative studies of virulence properties with the wild-type bacterium. Other advantages are that the technique is fast and is applicable to a wide range of bacteria, and importantly, it requires only a small number of host animals. However, this approach does come with its disadvantages. Transposon mutagenesis typically can only knock out or disrupt genes, so genes that suppress pathogenesis cannot be identified via Tn-Seq. Like other transposon methods, standard Tn-Seq can only be used to identify virulence genes with nonredundant functions, although variations of Tn-Seq can be used to identify synthetically lethal genetic relations (see above). Robust transposon mutagenesis systems are not available for all bacteria, although the Mariner-derived mini-transposon used for Tn-Seq almost randomly inserts into different sites on the chromosome.

Another complication is that the Tn-Seq approach entails a competition experiment (see chapter 8), in which multiple mutants are vying for growth in the same animal host. Secreted factors, such as toxins, can be complemented in *trans* from other members of the inoculated population, effectively masking the essentiality of certain virulence factors. Finally, determination of the fitness cost of knocking out a bacterial gene in an animal model is more complicated than for bacteria grown in culture. Bacterial generation time *in vivo* needs to be calculated, and bottlenecks caused by random stochastic processes, such as the elimination of bacteria by the immune system in animals, need to be considered. Fortunately, several clever methods have been devised to estimate bacterial generation time and bottleneck frequencies in animals.

RNA-Seq Technology to Identify *In Vivo*-Expressed Genes

RNA-sequencing (RNA-Seq), which involves next-generation Illumina sequencing of the whole bacterial and/or host transcriptome, as described in chapter 5 (see Figure 5-12), has emerged as the preferred method for genome-wide identification of bacterial genes that are expressed during infection. The assumption is that differentially regulated genes are important for survival in the host and/or virulence, and thus, these genes are good candidates for virulence factors and possible targets for drug or vaccine development. RNA-Seq technology can be used to analyze and profile the relative quantity of mRNA in a biological sample at any given time or condition, such as during an infection. Different approaches have been developed to determine the bacterial gene expression during exposure to host cells. Until recently, the usual RNA-Seq methods required first enrichment of the bacterial RNA by depleting the host cell content prior to analysis through selective lysis of the host cell followed by differential centrifugation or by sequence-specific removal of host transcripts from cell extracts before RNA analysis.

These enrichment methods have now been supplanted by **dual RNA-Seq technology**, which involves optimized sample preparation and quantitation of relative transcript amounts from pathogens and host cells together at the same time (Figure 9-9). Fluorescently labelled bacteria are allowed to infect host cells, and the host cells are then sorted by fluorescence-activated cell sorting (FACS) to enrich for a population of only bacterially infected host cells. Total RNA is then extracted from the bacterially infected host cells, and the highly abundant host and bacterial rRNAs are removed, before further processing to convert the remaining RNA into cDNA, which is then sequenced. The mixed sequencing reads are subsequently aligned with their respective genome templates *in silico* to yield the genome-wide relative transcription levels of the genes in each organism *in vivo*. Dual RNA-Seq has the capacity of allowing researchers to follow the dynamic interaction networks occurring within and between pathogens and hosts during the course of an infection.

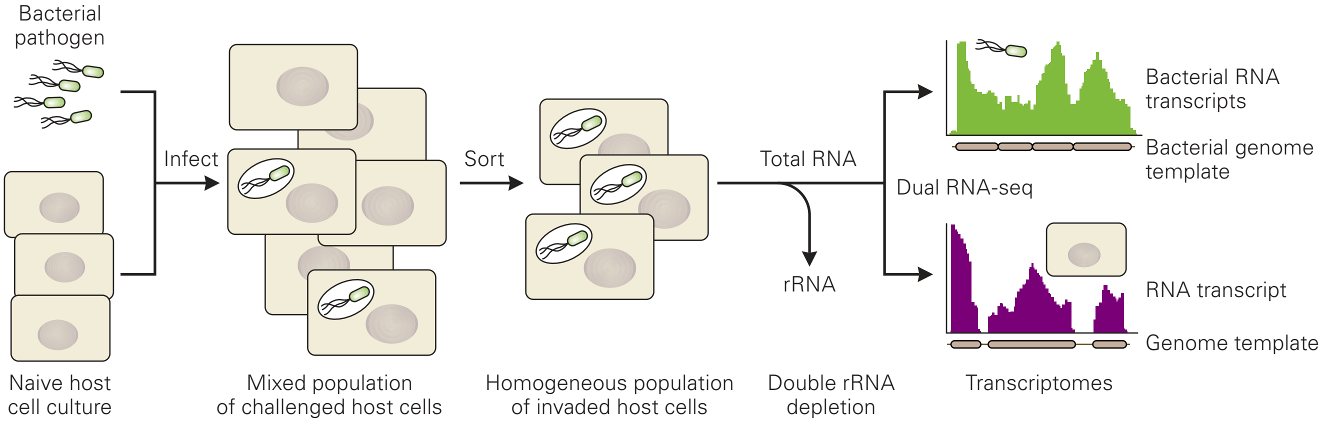

Figure 9-9. Using dual RNA-sequencing (RNA-Seq) transcriptomics approaches to identify genes regulated under different conditions. Transcriptomics approaches, such as RNA-Seq, can be used to determine bacterial mRNA expression levels under different conditions. In dual RNA-Seq approaches, gene expression of both pathogen and host is analyzed in parallel. Current methods involve a couple of enrichment steps: Fluorescently labelled bacteria are allowed to infect host cells, which are then sorted by fluorescence-activated cell sorting (FACS) to enrich for a population of host cells infected with bacteria. Total RNA is extracted from the bacterially infected host cells, and the highly abundant host and bacterial rRNA is removed. The remaining RNA is converted into cDNA and sequenced. The mixed sequencing reads are aligned with their respective genome templates *in silico* to determine the *in vivo* transcriptomes for both host and bacteria. Transcriptomes can readily be obtained for a time-course of infection, as well as for comparison of wild-type versus mutant bacteria.

Comparative Genomic Sequence Analysis for Identifying Virulence Genes

Current genome sequencing technologies (discussed in detail in chapter 5) have revolutionized how bacterial pathogens are interrogated nowadays. Tools and databases for exploring, analyzing, and comparing completed genomes can be found through the NCBI (https://www.ncbi.nlm.nih.gov/genome/microbes/).

Due to the increasing speed and efficiency of sequencing and assembling genomes at ever-decreasing costs, more and more genome sequences of pathogenic and nonpathogenic strains of the same bacterial species are becoming available. By comparing the genomes of these strains, it is possible to identify genes exclusively found in pathogenic strains but not in their nonpathogenic relatives. These strain-specific genes represent strong candidates for being virulence factors. In addition to virulence, similar comparative genomic approaches can also be applied toward identifying genes associated with other biological properties, such as adhesion, metabolite production, and antibiotic resistance.

Microbial genome sequence information and the ability to analyze the expression activity of every gene in a cell are

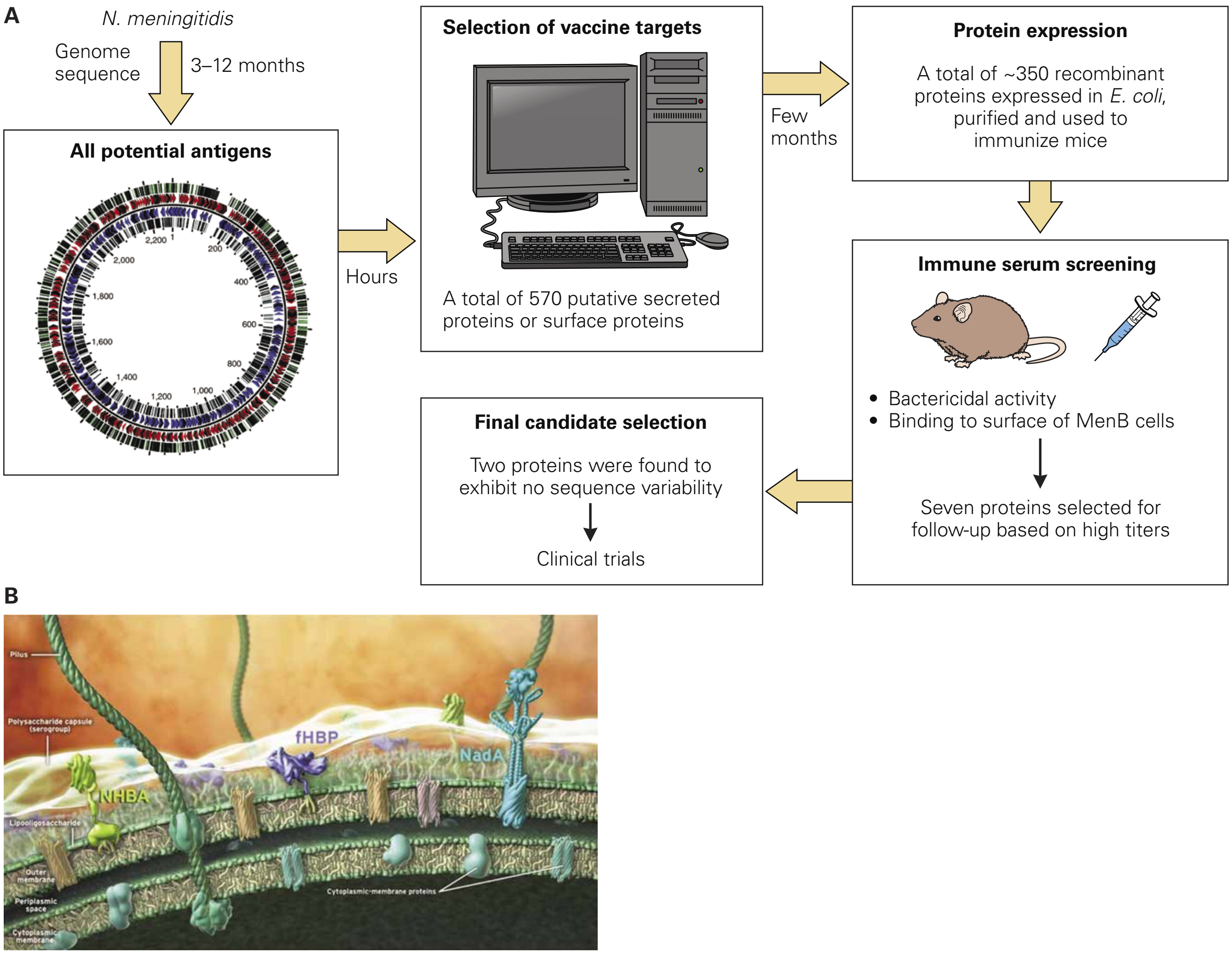
A
N. meningitidis
Genome sequence
3–12 months
All potential antigens
2,200
1
200
400
600
800
1,000
1,200
1,400
1,600
1,800
2,000
Hours
Selection of vaccine targets
A total of 570 putative secreted proteins or surface proteins
Few months
Protein expression
A total of ~350 recombinant proteins expressed in E. coli, purified and used to immunize mice
Immune serum screening
• Bactericidal activity
• Binding to surface of MenB cells
Seven proteins selected for follow-up based on high titers
Final candidate selection
Two proteins were found to exhibit no sequence variability
Clinical trials
B
Pilus
Polysaccharide capsule (serogroup)
fHBP
NadA
NHBA
Lipooligosaccharide
Outer membrane
Periplasmic space
Cytoplasmic membrane
Cytoplasmic-membrane proteins

Figure 9-10. Whole microbial genome sequencing to accelerate vaccine development. (A) Whole genome sequencing of a virulent serogroup B strain of *Neisseria meningitidis* (MenB) enabled accelerated vaccine development against meningococcal disease. A total of 28 surface proteins were found to induce antibodies with bactericidal activity. Two proteins were identified as exhibiting high sequence conservation and selected through this screening process for further vaccine development. Adapted from Fraser CM, Eisen JA, Salzberg SL. 2000. *Nature* **406:**799–803, with permission. Newly available methods allow for much faster sequencing of whole bacterial genomes, speeding the analysis up by months. **(B)** Schematic diagram showing the surface localization of the final three major protein components of the successfully licensed 4CMenB vaccine. NHBA, Neisserial heparin-binding antigen; fHBP, factor H-binding protein; NadA, Neisserial adhesin A. Reprinted from Serruto D, Bottomley MJ, Ram S, Giuliani MM, Rappuoli R. 2012. *Vaccine* **30 Suppl 2:**B87–97, with permission.

powerful tools that are accelerating vaccine development (Figure 9-10). Once a genome has been completely sequenced, all potential antigens can be identified using software analysis programs that predict proteins that might be secreted or expressed on the cell surface. These putative vaccine targets are then expressed as recombinant proteins in *E. coli* and the purified proteins are used to immunize mice. The immune sera are then screened for antibody binding to the bacteria and bactericidal activity. Those vaccine candidates that test positive in these assays are then further tested for their ability to provide protective immunity. In a recent example of the power of this technology, from a total of 570 putative secreted proteins or surface proteins identified from the genome sequence of *Neisseria meningitidis*, about 350 recombinant proteins were expressed in *E. coli* and used to immunize mice. Seven proteins were selected from the immune serum screen, and of those, two proteins were chosen as vaccine candidates for further clinical trials, and eventually three proteins were included in the final vaccine, 4CMenB.

Proteomics Approaches for Identifying Virulence Factors

Proteomics technologies, while still lagging behind genomics and transcriptomics technologies, are steadily advancing with improved analytical instrumentation with high-throughput separation and enhanced sensitivity capabilities (see chapter 5, Figure 5-13). There has been an explosion in recent years of vast amounts of structural and functional data and information about bacterial proteins and their analytical properties, as well as rapidly enlarging libraries of high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectral (MS), and crystallographic (protein data bank [PDB]) profiles. These tools are enabling the identification of proteins and changes in protein properties and profiles during infection. Below are a couple of areas where these technologies have been particularly helpful.

Protein Microarrays (Proteoarrays)

Although DNA/RNA-based microarray technology has been superseded by next-generation sequencing technologies, microarray technology has now been extended to proteins. Once the genome sequence of a pathogen has been determined, high-throughput PCR can be used to clone every protein reading frame of the pathogen into an expression vector. The corresponding proteins are then expressed using an *E. coli*-based cell-free *in vitro* transcription-translation system (which avoids the need to purify the expressed proteins), and the *in vitro*-expressed proteins are printed (spotted) onto nitrocellulose membrane microarrays to generate **protein microarrays (proteoarrays)**. Alternatively, the phage display approach mentioned below can be used to express the proteins from the pathogen, and then the pathogen displaying the protein on its surface can be attached to a well in a microtiter plate or some other matrix. It should be noted that because the *in vitro* expression system contains *E. coli* lysate, it is important to first remove the anti-*E. coli* antibodies that are normally present in human serum before performing the serological screening. This is not necessary for mouse serum, which lacks significant *E. coli* cross-reactivity.

Proteoarrays are often used to determine the antibody-binding profiles of serum (serological screening) from vaccinated humans or animals, which can then be used to identify cross-reactive antigens that might serve as potential vaccine candidates. The antibodies against a particular antigen or set of antigens can be covalently immobilized on a microarray glass surface to generate an antibody proteoarray (Figure 9-11). Here, biotinylated protein antigens present in samples obtained from naïve, vaccinated, infected, or convalescent patients can be screened for the antigen(s) of interest by first binding the biotinylated antigens to the antibody array and then treating with fluorescent dye-labeled streptavidin, which binds tightly to the biotin groups, and visualizing by fluorescence microscopy. In another example, whole-proteome microarrays of the sexually transmitted pathogen *Chlamydia trachomatis* were generated from genomic DNA using cell-free protein expression and PCR amplification directly on a chip (Figure 9-12). The microarrays displaying the bacterial protein antigens were then used for the immunoprofiling of patients to identify antigens binding to disease-related serum antibodies from patients testing positive for infection with *C. trachomatis* and/or cervical cancer.

In Vivo-Induced Antigen Technology (IVIAT)

An antibody-based genomic method that can be used to identify genes induced during human infections, yet avoids the use of animal infection models, is ***in vivo*-induced antigen technology (IVIAT)**. The method (illustrated in Figure 9-13) takes advantage of pooled serum from one or more patients who have been exposed to a particular pathogen and have developed a protective immune response. In the version of this method shown, the pooled serum is absorbed with whole cells or cellular extracts from bacteria grown *in vitro*. The remaining serum, which contains a subpopulation of antibodies reactive against *in vivo*-induced (IVI) antigens, is then used to probe a genomic expression library of *E. coli* clones that express genes from the pathogen. This step can also be performed by using **phage display**, which incorporates regions from the pathogen proteins into the surface coat protein of a bacteriophage. This display method lends itself to high-throughput screening methods. Those clones expressing proteins that are cross reactive with the remaining antibodies in the serum are identified as putative IVI antigens that might generate protective immune responses, and hence are potential vaccine candidates. Potential candidate proteins are then purified and inoculated into an animal model, if available, to determine whether they are antigenic. If antibodies are produced, the vaccinated animals can then be challenged with the pathogen to learn whether the antibodies are protective against the infection.

In addition, IVIAT has been useful for identifying virulence factors involved in human infection and for identifying virulence factors of human-specific pathogens for which there is no suitable animal model. Finally, in some approaches related to IVIAT, such as antigenic fingerprinting, the initial serum is not pre-absorbed to cells or extracts grown *in vitro*. This approach identifies antigenic surface proteins that are expressed both *in vitro* and *in vivo*, including essential surface proteins involved in cell division and signaling. IVIAT technologies have also been combined with protein microarrays mentioned above for determining the complete antigen-specific humoral immune response profile from vaccinated or infected humans and animals.

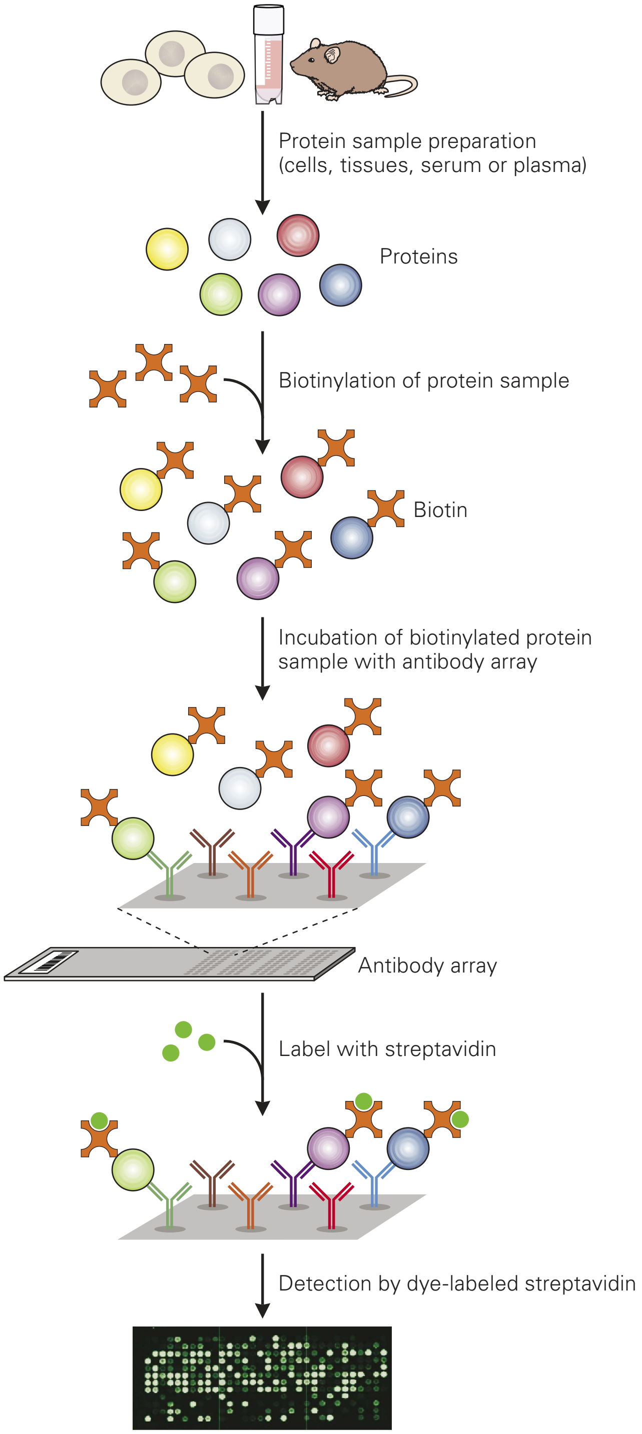
Protein sample preparation
(cells, tissues, serum or plasma)
Proteins
Biotinylation of protein sample
Biotin
Incubation of biotinylated protein
sample with antibody array
Antibody array
Label with streptavidin
Detection by dye-labeled streptavidin

Figure 9-11. Protein microarray technology to identify *in vivo*-expressed proteins. Protein microarray technology can be used for qualitative and/or comparative protein expression profiling to identify potential virulence factors. In this approach, protein samples are prepared from healthy and infected or convalescent host cells, tissues, or serum. The proteins are biotinylated and then incubated with an antibody proteoarray, which is generated from antibodies against particular antigens of interest. The biotinylated proteins that bind to the antibody spots are detected and/or quantified using fluorescence microscopy by fluorescent dye-labeled streptavidin.

The Importance of Understanding Bacterial Physiology

In retrospect, a critical experiment done on *S. typhimurium* presaged what is now being learned from genomics and other ultra-modern approaches. The investigators introduced transposons nearly randomly into a virulent strain of *S. typhimurium*. For purposes of this study, the investigators defined "virulence" as the ability of *S. typhimurium* to grow inside macrophages. They screened thousands of transposon-generated mutants for the ability to survive and grow inside macrophages. Their results led them to conclude that at least 200 genes (over 5% of the chromosome) were involved in the ability of *S. typhimurium* to survive in macrophages, and these genes were scattered all over the bacterial chromosome. Today, comparisons of the genome sequences of, say, *S. typhimurium* and *E. coli* K12 (an avirulent *E. coli* strain) or of *S. typhimurium* and *S. typhi* (the cause of typhoid fever in humans) show that the differences are many and are indeed scattered throughout the chromosome. Clearly, virulence is multifactorial. In addition, it has become clear that genes important for virulence in one strain may also be located in a nonvirulent strain of the same species, and hence the assumption that only virulent organisms contain virulence genes is flawed.

More often than not, the genes identified by the approaches described in this chapter have proven to be genes that would be considered housekeeping genes or stress response genes. Perhaps what this is indicating is that it is the entire physiology of the bacterium, not just a few genes, that is important. Indeed, introduction of a mutation in a gene that confers a certain phenotype is just the beginning of understanding the function of that gene's product, whether it functions as a protein or a regulatory RNA. Typically, most gene products act in concert with other gene products in

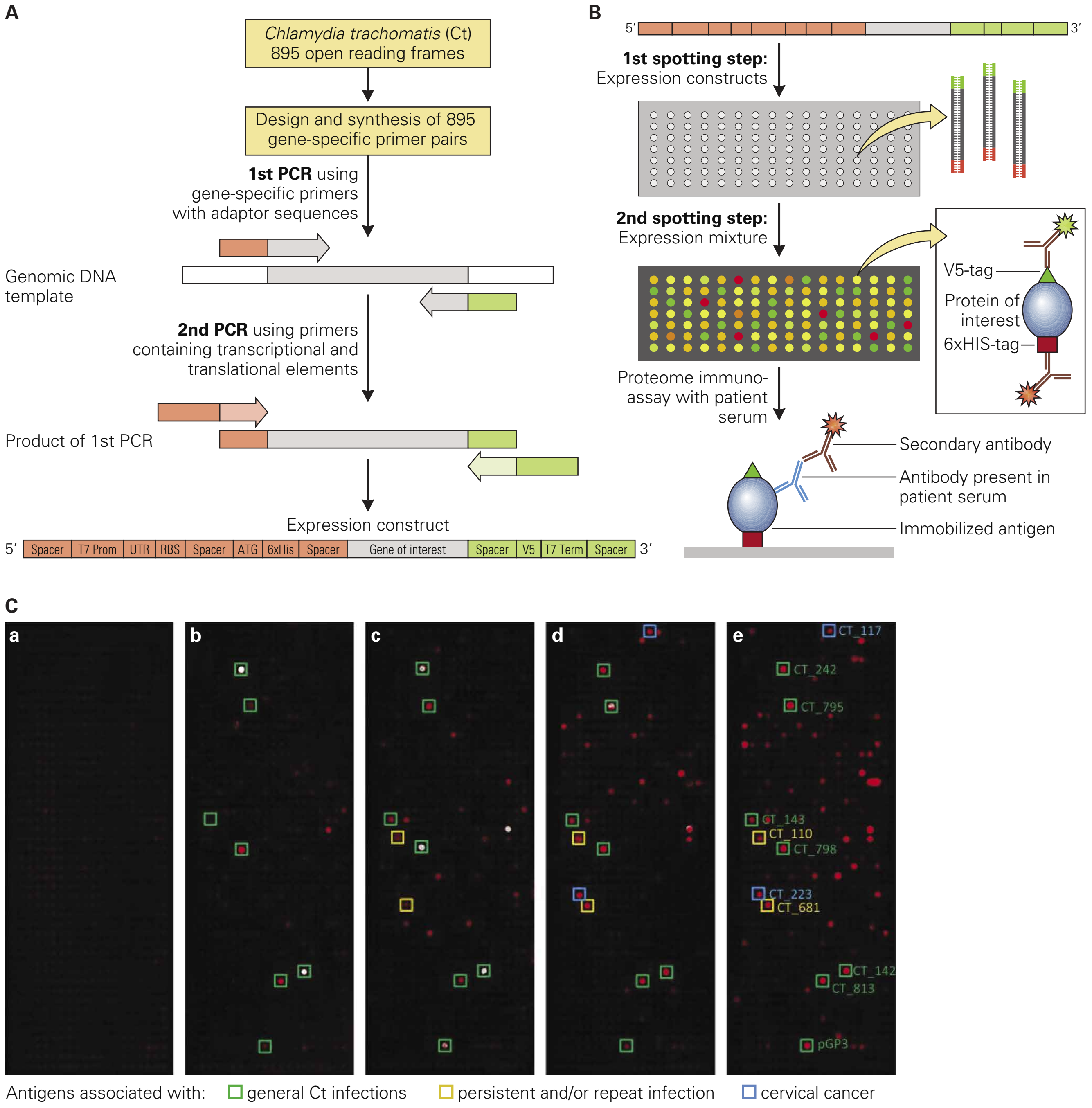
A
Chlamydia trachomatis (Ct)
895 open reading frames
Design and synthesis of 895
gene-specific primer pairs
1st PCR using
gene-specific primers
with adaptor sequences
Genomic DNA
template
2nd PCR using primers
containing transcriptional and
translational elements
Product of 1st PCR
Expression construct
5′
Spacer
T7 Prom
UTR
RBS
Spacer
ATG
6xHis
Spacer
Gene of interest
Spacer
V5
T7 Term
Spacer
3′
B
5′
3′
1st spotting step:
Expression constructs
2nd spotting step:
Expression mixture
V5-tag
Protein of
interest
6xHIS-tag
Proteome immuno-
assay with patient
serum
Secondary antibody
Antibody present in
patient serum
Immobilized antigen
C
a
b
c
d
e
CT_117
CT_242
CT_795
CT_143
CT_110
CT_798
CT_223
CT_681
CT_142
CT_813
pGP3
Antigens associated with:
🟩 general Ct infections
🟨 persistent and/or repeat infection
🟦 cervical cancer

Figure 9-12. Generation of *Chlamydia trachomatis* whole-proteome microarrays for immunoprofiling of patients. (A) Based on the genome sequence from *C. trachomatis* (Ct), gene-specific primer pairs with adapter sequences were designed for all 895 open reading frames of Ct. The resulting PCR products containing the genes of interest were further amplified with a second set of PCR primers that overlap with the complementary adaptor sequences of the first primer set and that also have additional transcriptional and translational regulatory elements for expression of the proteins: T7 promoter (T7 Prom), untranslated region (UTR), ribosome binding site (RBS), start codon (ATG), T7 terminator (T7 Term), N-terminal hexahistidine (6xHis) affinity tag for protein purification, and C-terminal V5 epitope tag for labeled antibody recognition. **(B)** The expression constructs were transferred to Ni^{2+}-nitriloacetic acid (NTA) chelation matrix-coated slides. The expression reaction mixture was then spotted on top of the first spots, and proteins were expressed directly on the slide. Efficiency of protein expression was determined using fluorescent dye-conjugated antibodies against the V5 and 6xHis tags. The expressed proteins immobilized to the Ni^{2+}-NTA matrix via the 6xHis tags were then exposed to serum antibodies, and antibodies present in the patient's serum that bound to any of the immobilized proteins were visualized and quantified using a fluorescent dye-conjugated anti-human secondary antibody. **(C)** Fluorescent image results from proteome microarray immunoprofiling using 5 pools of 5 patients' sera: a = Ct-uninfected women; b = cancer-free, Ct-infected women <25 years old; c = cancer-free, Ct-infected women >40 years old; d, e = 2 different pools from Ct-infected cervical cancer patients. Examples of Ct antigens associated with different conditions: green boxes = general Ct infections; yellow boxes = potentially persistent and/or repeat infections; blue boxes = cervical cancer. Panels A and B adapted from, and Panel C reprinted from Hufnagel K, Lueong S, Willhauck-Fleckenstein M, Hotz-Wagenblatt A, Miao B, Bauer A, Michel A, Butt J, Pawlita M, Hoheisel JD, Waterboer T. 2018. *Sci Rep* **8:**7503, with permission.

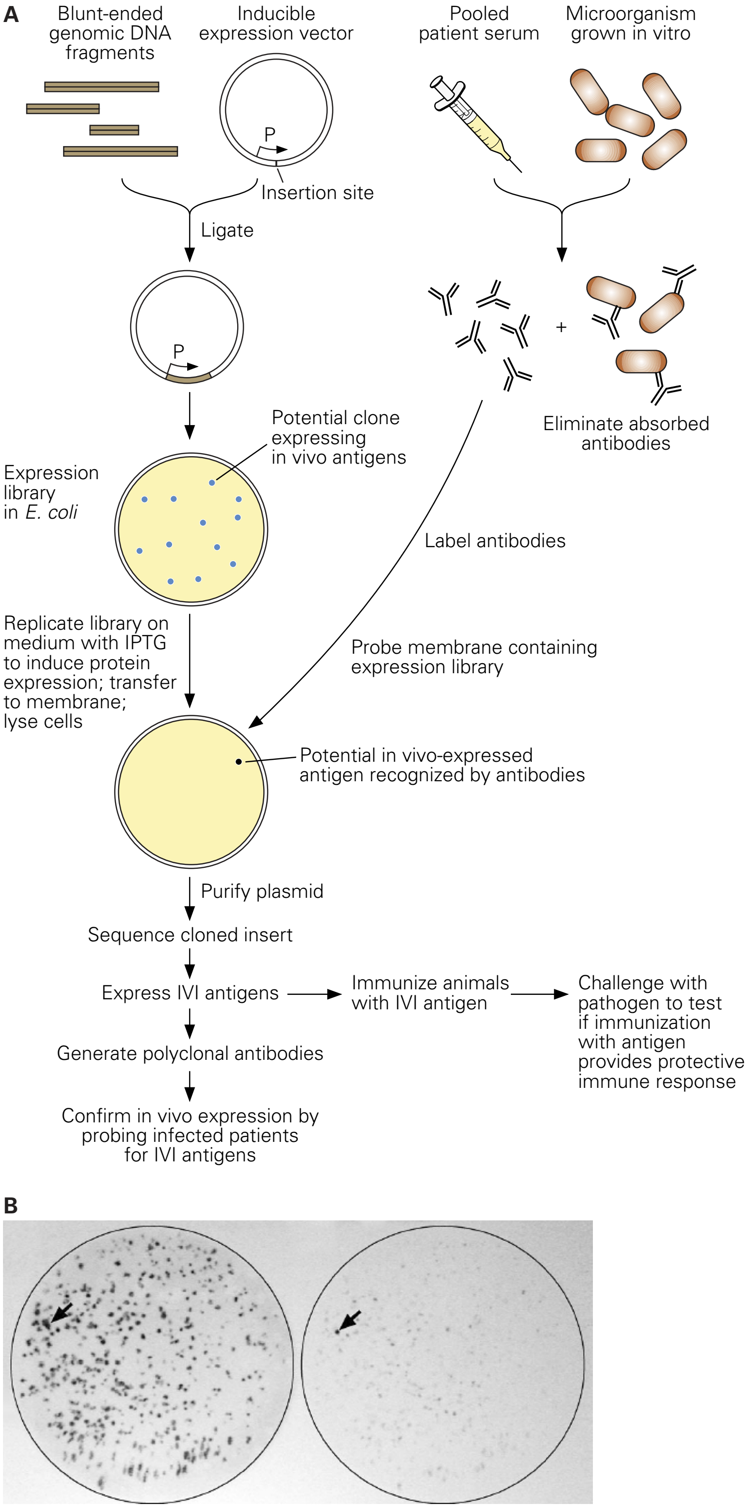
A
Blunt-ended genomic DNA fragments
Inducible expression vector
P
Insertion site
Pooled patient serum
Microorganism grown in vitro
Ligate
P
+
Eliminate absorbed antibodies
Potential clone expressing in vivo antigens
Expression library in *E. coli*
Label antibodies
Replicate library on medium with IPTG to induce protein expression; transfer to membrane; lyse cells
Probe membrane containing expression library
Potential in vivo-expressed antigen recognized by antibodies
Purify plasmid
Sequence cloned insert
Express IVI antigens
Immunize animals with IVI antigen
Challenge with pathogen to test if immunization with antigen provides protective immune response
Generate polyclonal antibodies
Confirm in vivo expression by probing infected patients for IVI antigens
B

Figure 9-13. ***In vivo*-induced antigen technology (IVIAT). (A)** Sera from patients who have experienced an infection caused by the pathogen under study are pooled and exhaustively absorbed with cells of the pathogen grown *in vitro*, leaving antibodies against antigens that are expressed only *in vivo* (step 1). An expression library of the pathogen's DNA is generated in a suitable host (step 2) and clones are probed with the absorbed serum (step 3). Reactive clones, which are producing antigens that are expressed during a natural infection but not during *in vitro* cultivation, are purified and their cloned DNA sequenced (step 4). Genes are identified in this fashion as encoding *in vivo*-induced (IVI) antigens. These antigens are purified and used to verify that the IVI antigen is expressed by the pathogen during an infectious process (step 5). This can be done using fluorescent-labeled antibodies raised against the purified IVI antigen to probe biological samples taken from infected patients. (**B**) Screening of a partial genomic expression library using IVIAT. The library was created using size-selected genomic DNA fragments isolated from the periodontal pathogen *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans*, the etiological agent of localized juvenile periodontitis (LJP). The DNA was digested with a restriction enzyme, cloned into an expression plasmid and transformed into *E. coli*. The resulting library was then grown on agar plates and the recombinant proteins were expressed. Duplicate colony blots of the induced expression library were prepared on nitrocellulose membranes and probed with either (a) pooled unabsorbed serum from LJP patients or (b) the same pooled serum absorbed with *A. actinomycetemcomitans* grown *in vitro*. Reactive clones (indicated by an arrow) are identified by sequencing and the proteins are purified and further studied. Adapted from Handfield M, Brady LJ, Progulske-Fox A, Hillman JD. 2000. *Trends Microbiol* **8**:336-339, with permission.

what are often referred to as interaction networks that make up complex cellular processes. Ideally, clues gained from Tn-Seq or RNA-Seq technologies should be followed up with other biochemical, analytical, or microscopy studies to determine the functional role of the gene in the context of its interaction network. Proteomics and metabolomics technologies (discussed in chapter 5) are helpful in this area.

Another striking finding that has come out of the genome sequencing data is that about one-third of all the genes are not recognizable (i.e., they code for proteins of unknown function). The majority of the genes that have been identified as putative virulence genes have no significant matches in the known databases. (How "significant" is defined varies somewhat from one scientist to another, but usually means that the amino acid sequence identity is at least 20%.) In addition to the sequences that are not recognized at all are the ones for which the tentative identification is questionable. For instance, a deduced amino acid sequence that contains a consensus ATP binding site may be identified as an ATPase, when it actually has a completely different function. Again, about one-third of the identified genes often fall into this category. Ultimately, it is going to take a new initiative to advance our knowledge of bacterial physiology to the point that the sequence information becomes truly decipherable. More than that, the interactions between different pathways remain to be elucidated. Importantly, the expression of different virulence genes and the relationships of the proteins they encode are highly interactive. How are we to understand this second layer of complexity, beyond the function of gene products to the interaction of these products with those of other genes?

Bacterial physiology will be the field most likely to get at the essence of what it means to be a living organism. For instance, the genome sequence of a tiny bacterium called *Mycoplasma genitalium*, which contains about 300 genes, currently stands as the simplest genome of a free-living organism. However, over 100 of these genes were not recognizable as having a known function. What is exciting about this small genome is that it raises the hope that if scientists could understand the function and interactions of this small number of gene products, they might finally have an insight on what defines "life," at least at the single-cell level. Again, these future insights must come from more sophisticated studies of bacterial physiology, coupled with high-throughput methods.

Collections of knockout mutations have been constructed in every nonessential gene of *E. coli*. In fact, two insertion mutations were constructed in every gene, each imparting resistance to two different antibiotics. Therefore, it is theoretically possible to construct double mutants to test the genetic interactions between every pair of nonessential genes in *E. coli*, but the numbers would be very large (e.g., just 20 knockouts would lead to 190 unique pair-wise combinations). However, high-throughput methods coupled with robotic handling is making this approach possible, and from these studies we are beginning to learn the possible functions of many of the unknown genes mentioned above. These approaches are also beginning to be applied to pathogens, such as *Streptococcus pneumoniae*. Finally, new genome-wide methods using CRISPR-Cas9 interference to turn off bacterial genes show great promise in determining the functions, interactions, and redundancies of genes in a broad range of pathogens (see chapter 7). In addition, mobile CRISPR-Cas9 methods are being developed to allow the transfer of CRISPR-Cas9 interference into pathogenic and microbiome bacteria that lack facile genetic systems.

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Questions

1. Scientists have proposed a molecular version of Koch's postulates for associating a bacterium with a disease. What would such postulates look like and what would be their limitations?
2. The genomes of a number of bacterial pathogens have now been completely sequenced. How might the availability of this information affect the definition of virulence factors?
3. Critique the statement: If you compare genomes of a pathogen and a closely related nonpathogen (e.g., *S. typhimurium* and *E. coli* K12), the differences will be the virulence genes.
4. Why are *in vivo* expression technologies continuing to turn up housekeeping genes rather than the "virulence genes" the researchers who developed these methods originally envisioned?
5. If you were working with a newly isolated bacterium, what would you have to be able to do before you could use gene fusions to identify virulence genes? Do these same restrictions apply to microarrays? What would you need to have to use microarray technology?
6. If you have the genome sequence of a bacterial pathogen, would you still need to clone genes or does cloning become obsolete?
7. What basic assumption is made about the regulation of potential virulence factors when using Tn-Seq or RNA-Seq?
8. Suppose you perform an RNA-Seq experiment comparing RNA samples prepared from a bacterium isolated from the blood of an infected animal to that grown in rich tissue culture medium *in vitro*. You detect changes in the relative transcript amounts of several genes for the bacterium grown in blood versus culture medium. How do you interpret these results? What does it tell you about the virulence of this bacterium?

Solving Problems in Bacterial Pathogenesis

1. Comparison of the genomes of wild-type bovine tuberculosis strains of *Mycobacterium bovis* and contemporary *M. bovis*-derived Bacille Calmette-Guerin (BCG) vaccine strains with that of a virulent reference strain of *M. tuberculosis* indicated that almost 100 *M. tuberculosis* genes were found to be "missing" from all of the *M. bovis* and BCG isolates that were used for making the vaccines. Describe how you might isolate and identify those genes that are unique to *M. tuberculosis*.
2. You are a researcher working for the USDA. After two years of effort, you have isolated in pure culture a new, highly virulent bacterium from duck feces that is responsible for several major outbreaks of deaths in mammalian wildlife from contaminated pond water in the South. Based on 16S rRNA sequence comparison, you have determined that this new bacterium is distantly related to the Gram-negative bacterium *Vibrio cholerae*, and you have named the new strain *Vibrio birdsii*. You

have subsequently determined that *V. birdsii* is sensitive to tetracycline (i.e., it cannot grow in its presence), but it is resistant to ampicillin. You obtained a *Vibrio* plasmid that you then used to construct a suitable *E. coli* shuttle vector for *V. birdsii,* which you could use for genetic manipulation in *E. coli* and then transfer into *V. birdsii* by transformation. You have also determined that guinea pigs, which have good innate immune systems, are an excellent animal model for the disease, which results primarily from uptake through drinking contaminated water, followed by colonization of the gut and bloody diarrhea, followed by invasion of intestinal cells with spreading to lymph nodes and the spleen, and then death by dehydration and/or organ failure. You are interested in identifying virulence factors associated with disease in guinea pigs. You have decided to use Tn-Seq as a strategy to identify these virulence factors. After infection and harvesting of the intestines, spleen, and lymph nodes of the guinea pigs, you have identified 10 genes that are likely involved in growth and another 6 that might be involved in virulence.

a. Draw a diagram of the shuttle vector and transposon that you constructed. Be sure to label all the key features (genes, promoters, etc) that are necessary for them to work for *V. birdsii* in order to use in your studies.
b. How do you interpret the results from the initial screen?
c. Describe how you would verify that the putative virulence factors identified by your method are indeed involved in pathogenesis. Be sure to state what specific criteria must be satisfied.

3. You are a researcher working for the National Wildlife Health division of the U.S. Geological Survey. After two years of effort, you have isolated in pure culture a new, highly virulent bacterium from fish that is responsible for several major outbreaks of fish deaths along the coasts and the Great Lakes. Based on 16S rRNA sequence comparison, you have determined that this new bacterium is distantly related to the spirochete *Cristispira clone*, which is thus far noncultivatable, and you have named it *Cristispira fisherii.* You have subsequently determined that *C. fisherii* has an unusual polysaccharide capsule and that it is sensitive to chloramphenicol, tetracycline, and erythromycin, but is resistant to ampicillin, penicillin, and gentamicin. You have also determined that it contains two similar circular chromosomes and three endogenous plasmids, one of which has some homology with plasmids from Gram-negative bacteria. You have used this plasmid to construct an *E. coli* shuttle vector for *C. fisherii,* which you can use for genetic manipulation in *E. coli* and transfer into *C. fisherii* by electroporation. You have also determined that Zebra fish, which have good innate immune systems, are an excellent animal model for the disease, which results primarily from uptake through the gills into the fish lungs, followed by invasion and dissemination through the body, and then death.

   a. You are interested in identifying virulence factors associated with infection in fish. Considering all of the above information, describe *in detail* a strategy that you might use to identify these virulence factors. Be sure to provide a rationale for your choice of strategy, the appropriate reagents that you will need to use, the overall experimental design, and how you will determine the identity of the virulence factors.
   b. Describe how you will verify, using the tools at hand, that the putative virulence factors identified by your method are indeed involved in pathogenesis.
   c. From your screening, you identified two genes encoding putative virulence factors, which you have named *cff1* and *cff2* for *C. fisherii* factors 1 and 2. You have created mutant strains with deletions in these two genes. The wild-type bacterium has ID_{50} and LD_{50} values of 10. When administered to the Zebra fish via the water, both mutant strains Δ*cff1* and Δ*cff2* have ID_{50} and LD_{50} values of 10^7. But, when injected into the dorsal muscle of the fish, Δ*cff1* has an ID_{50} value of 10^2 and an LD_{50} value of 10^4, while Δ*cff2* has an ID_{50} value of 10^2 and an LD_{50} value of 10. Provide an explanation for these findings.

4. From infected rabbits, you have isolated a new, highly virulent Gram-positive bacterium related to *Listeria monocytogenes*, which you have named *Listeria leporine*. Pathologic findings are most prominent in the intestine and spleen. Clinical signs are generally mild or absent in adult healthy animals, but you find that young and old animals have symptoms of increased thickness of the lining of the gut due to proliferation of epithelial cells and swelling of the lymph nodes and spleen. Those animals often succumb to systemic infection, including brain lesions and death in about 70% of the cases.

   a. You wish to better understand the pathogenesis of the disease and to identify potential virulence factors of *L. leporine*. But, because these results are rather urgent, you decide to use the available genome sequence of the related *L. monocytogenes* to conduct a comparative genome analysis. Describe how you would go about conducting this study. Be sure to include all of the reagents and steps you would need to accomplish this task.
   b. From your comparative genome analysis, you identified seven genes encoding putative virulence factors, which you have named *llp1* through *llp7*. Two of the genes (*llp1* and *llp2*) were found adjacent to each other

on a two-gene operon, the genes *llp3* and *llp4* were part of an operon consisting of four genes, the gene *llp5* was part of an operon consisting of five genes, and the other two genes (*llp6* and *llp7*) were found on separate single-gene operons. Describe *in detail* how you would verify using a young rabbit infection model that those seven encode actual virulence factors. Be sure to state the criteria that would need to be satisfied. Be sure to include a description of all the reagents, conditions, and experimental procedures you would need to use, as well as your rationale.

   c. You would like to know at which point during the *in vivo* infection process each of these putative virulence genes gets turned on. Describe an experiment that you could perform to determine this.

5. A new Gram-positive pathogen that causes skin rashes, headache, stiff neck due to swelling of the meninges (connective tissue layers covering the brain and spinal cord), and temporary memory loss following intense periods of concentration has recently been identified from the human "bookworm." This novel microbe is part of the commensal skin microbiota of most college students, but frequent outbreaks of disease occur in many students during exam periods. The pathogen appears to be host specific and hence there is no experimental animal infection model available. However, the bacterium can be isolated from infected skin lesions of the hosts and can be cultivated in pure culture on a complex human serum-containing medium in the laboratory that contains the human stress hormones cortisol, norepinephrine, and growth hormone. PCR amplification, sequencing, and phylogenetic comparisons of the 16S rRNA gene indicate that the organism is related to *Staphylococcus epidermidis* species and so it was named *S. bookwormii*. You would like to help out your fellow bookworms by studying the pathogenesis of this pathogen and developing a vaccine.

   a. Briefly describe how you would embark on identifying *in vivo*-expressed genes that are produced by *S. bookwormii* during infection. Be sure to provide a rationale for your choice of strategy, the overall experimental design, and how you will determine the identity of the putative virulence factors.
   b. Briefly describe how you will verify that the putative virulence factors identified by your method are indeed involved in pathogenesis.
   c. Briefly describe how you would determine whether these putative virulence factors are indeed viable vaccine candidates that can be used to generate a protective immune response.

10

IN THIS CHAPTER

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CHAPTER 10

Identification of Virulence Factors

MOLECULAR APPROACHES FOR HOST FACTORS

Anyone who has ever read a mystery novel or watched a mystery program on TV knows that criminals have an MO (*modus operandi*) that needs to be understood before the evildoer can be identified and arrested. An important part of that MO is how the perpetrator attacks the victim. In the area of bacterial pathogenesis, the identification of bacterial virulence factors, together with an understanding of how these factors are designed to combat human defenses against infection, is tantamount to defining the MO of a bacterial pathogen. But also critical is the evidence created by or left behind by the perpetrator at the crime scene, which in this case is the host body. As with human criminals, there is no single MO for all bacteria, and indeed, the host signatures associated with certain infections can be used to identify and discriminate among different pathogenic bacteria. The detection and interrogation practices that establish this MO for different bacterial pathogens occur increasingly at the molecular level and at the host-microbe interface.

Comparative Approaches to Identify Host Factors Required for Infection

Modern technological advances have revolutionized the hunt for host factors important for infection. These technologies have enabled access to the complete genome sequences of humans, nonhuman primates, and multiple strains of different nonprimate animal models, plus the ability to rapidly and inexpensively determine the sequence of particular genes or chromosome loci and to determine the transcriptional, translational, and metabolic profiles under different *in vivo* conditions and among different hosts. The ability to genetically manipulate animal hosts (i.e., to generate **transgenic animals**) has greatly expanded the potential insights that can be gained from *in vivo* models of infection (the stand-in for the "victim" in our crime scene analogy). In addition, we now have a number of powerful technologies that enable *in vivo* visualization and monitoring during the infection process (essentially capturing the microbial "perp" red-handed, so to speak, in our crime scenario).

Transgenic Animal Models

The most common type of transgenic animal is the gene knockout mouse, where a particular gene is deleted, truncated, or inactivated. However, it is now also possible to have gene knockin mice, where a particular gene is introduced; gene replacement mice, where a particular gene is replaced with that of another animal or human gene or with a mutant version of the gene; and gene-edited mice, where the target gene is edited through CRISPR-Cas9 gene-editing technology (see chapter 7). The ever-expanding library of mouse mutants (Table 10-1), resulting from spontaneous or engineered mutations with defined phenotypes such as varying degrees of immunodeficiency, provides a powerful set of experimental animal models and tools for studying bacterial disease processes.

Another common strategy nowadays is to use humanized mice, in which the human version of a particular gene is introduced into the animal, such that the animal model of infection more closely resembles that of a human infection. An important application of transgenic animal technology is its use for determining mechanisms by which host genes are differentially regulated during establishment of an infection and subsequent disease progression and recovery. While transgenic mice remain the most common animal used for these types of studies, other animal species, including rats, rabbits, poultry, fish, swine, ruminants, nematodes (roundworms), and insects, are increasingly being utilized due to their unique features for particular applications or their suitability as infection models for certain diseases.

Immunodeficient Transgenic Animal Models. One common strategy for understanding infection processes is to compare the host responses in immunodeficient transgenic animals upon infection with wild-type bacteria, and then compare the responses to those obtained with mutant bacterial strains that have specific deletions in particular bacterial virulence factors. This approach allows researchers to better understand the role and interplay of the bacterial and host virulence factors in pathogenesis.

For example, **Nramp1 (natural resistance-associated macrophage protein-1)** is a host factor that provides resistance against several intracellular pathogens, such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and

Table 10-1. Examples of animal infection models

Infant mice have immature immune systems, so they are more susceptible to infection.
Irradiated mice are immunocompromised because their immune cells have been destroyed through radiation treatment, so they are more susceptible to infection.
Neutropenic mice are defective in the ability to produce neutrophils (PMNs), so they are more susceptible to infection. Neutropenia can be congenital due to a defect in the neutrophil elastase gene or acquired through chemical treatment (chemotherapy) or autoimmunity (anti-neutrophil antibodies).
NOG and NSG (NOD SCID) mice are a next generation of severely immunodeficient mice that are the result of crossbreeding of NOD, SCID, and IL-2 receptor γ-chain null mice. These mice have multiple defects in adaptive immunity, including the inability to generate T cells, B cells, and NK cells and reduced function in macrophages and dendritic cells. NOG and NSG mice have defects in innate immunity due to a mutation in the NOD gene. NSG mice also have defects in the PRKDC gene, which encodes a protein involved in V(D)J recombination during T and B cell development.
Nude mice are genetically defective in the FOXN1 gene, which is involved in development of a functional thymus and consequently in the ability to produce T cells. Nude mice are more susceptible to infection. A phenotype resulting from this genetic defect is the lack of body hair, hence the name "nude" mice. These mice can receive many different types of foreign material, including genes, cells, and tissues, without mounting an immune response that leads to rejection.
SCID mice are genetically defective in the ability to produce functional B cells and T cells, so they are more susceptible to infection but good for examining innate immunity. SCID mice can result from defects in a number of possible genes, but most cases are due to X-linked mutations in the common γ-chain of the interleukin receptor.
Transgenic mice are genetically defective in specific immune cells or immunity genes, so they are more susceptible to infection. Today, there are large resources of these types of transgenic mice available for studies. One popular strategy is to introduce human versions of genes to generate "humanized" transgenic mice. Other strategies are to knockout, knockin, replace, or gene-edit the mice for tailor-made functions.
Gnotobiotic (germ-free) animals are born under aseptic conditions, such as through removal from mother via Caesarean section, and raised in a germ-free environment, such as a sterile incubator. Because of the absence of resident microbiota, they have severely underdeveloped mucosa-associated lymphoid tissue (MALT). They also have no partial immunity due to prior exposure to any microbes, so they are "truly" naive (unimmunized) animals.
Specific-pathogen-free (SPF) animals are raised in an environment free of particular defined pathogens but are otherwise exposed to microbes in the environment, including other potential pathogens. Use of SPF animals for laboratory experiments ensures that current or prior exposure to the specified pathogen does not or has not occurred, which could interfere with interpretation of data from an experiment. Verification of SPF is usually performed through detection of antibodies in serum or by PCR analysis.

Mycobacterium bovis. Mice with a mutant *Nramp1* gene are defective in metal ion transport and function in phagocytic cells, such as macrophages and neutrophils. Researchers found that these mice require neutrophils to control wild-type *S.* Typhimurium infection. Similarly, an *Nramp1* mutation resulted in exacerbation of disease outcome, when the mutant animals were infected with an attenuated *M. bovis* Bacillus Calmette-Guérin (BCG) vaccine strain, pointing to important roles for the *Nramp1* protein in controlling infection with these two pathogens that reside within phagocytes.

Studies using cytokine receptor knockout mice have been critical for showing the importance of the cytokines IFNγ and TNFα in development of live vaccines. IFNγ receptor knockout mice are susceptible to infection with an attenuated vaccine strain of *S.* Typhimurium carrying a deletion in the *aroA* gene *(ΔaroA)*. On the other hand, although TNFα receptor knockout mice are not killed by the Δ*aroA* strain of *S.* Typhimurium, they are defective in their ability to develop immune memory after vaccination. When these mice are challenged with wild-type bacteria after vaccination with the Δ*aroA* strain, they are not protected from infection.

In another example, immunodeficient mice were used to determine the *in vivo* role of the *Bordetella* adenylyl cyclase toxin (CyaA) in pathogenesis. In wild-type mice that are immune competent, both wild-type bacteria and mutant bacteria with a deletion in the *cyaA* gene (Δ*cyaA*) readily colonized the respiratory tracts of the animals but produced no overt symptoms of disease. However, in mice deficient in T cells and B cells of the adaptive immune system (e.g., **SCID** [**severe combined immunodeficient**] or **RAG-1** [**recombination-activating gene 1**] knockout mice; see Table 10-1), infection with wild-type bacteria led to death of the mice. In contrast, although the Δ*cyaA* mutant still colonized these immunodeficient mice, it was not a lethal infection, suggesting that the adaptive immune response of the host is important in providing protection against the CyaA toxin. *In vitro* studies had previously suggested that CyaA would target neutrophils and the innate immune response early in infection. However, both wild-type and the Δ*cyaA* mutant bacteria killed **neutropenic** mice (mutant mice with low neutrophil counts or mice chemically treated to deplete their neutrophils, see Table 10-1). Together, these results showed that neutrophils and innate immunity are important for controlling the early stages of infection before the adaptive response kicks in, regardless of whether toxin is produced or not, while adaptive immunity is important for protecting against toxin-mediated disease.

Another approach that has been used to generate transgenic animals with defects in particular genes, such as those that are involved in certain developmental stages or in certain immune responses, is to remove cells expressing developmental or immune-directing genes by **genetic ablation**. Transgenic animals carrying a cytotoxic gene, such as the catalytic domain of diphtheria toxin (DT) that blocks protein synthesis and results in cell death (see chapter 12), under the control of a lineage-specific enhancer or a drug-inducible promoter, can be programmed to remove (**ablate**) cells that express the toxin protein under the desired conditions. Because mice lack receptors for DT, they are normally resistant to DT treatment. Refinement of the genetic ablation technique now utilizes a lineage-specific enhancer to first express in mice the human-specific receptor for DT to generate transgenic mice that express the receptor only in the desired tissues, followed by injection of DT to selectively ablate only those cells that express the toxin's receptor (Figure 10-1).

An advantage of the genetic ablation approach is that the animals grow normally until they reach a predefined point, at which time they can be induced to become deficient in a particular gene or genes, or in a particular type of

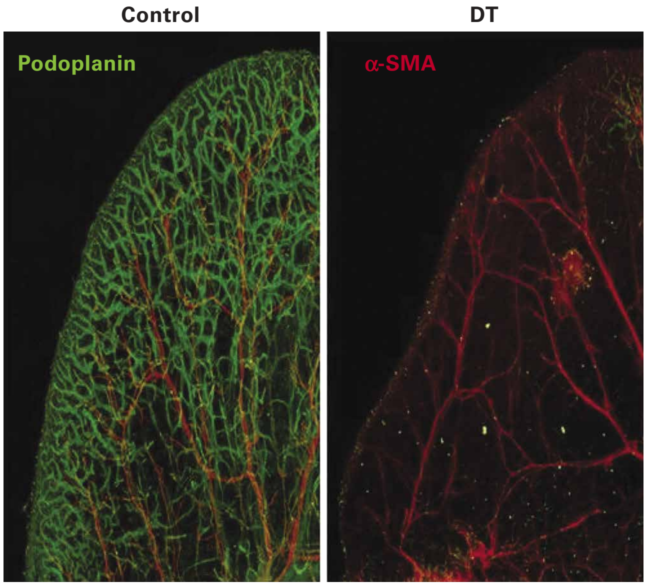


Figure 10-1. Diphtheria toxin-mediated ablation of lymphatic endothelial cells. Unlike humans, mice are not susceptible to diphtheria toxin (DT) because they lack the human-specific cell surface receptor for DT (heparin-binding epidermal growth factor-like growth factor precursor, proHB-EGF). Transgenic mice were generated that only express proHB-EGF under the control of a lymphatic-specific promoter. Localized subcutaneous injection of DT into the hind limb of the mice resulted in ablation of only the lymphatic system of the hind limb. Shown are microscopic images of dual immunofluorescence stained ear skin tissue slices obtained from mice injected with buffer control (left panel) or DT (right panel). One week after DT treatment the skin tissue images showed nearly complete ablation of the entire lymphatic vascular system, including the capillary lymphatic vessels (stained with green-dye labeled antibodies against podoplanin, a small mucin-like transmembrane protein selectively expressed in lymphatic endothelial cells) and the collecting lymphatic vessels (stained with red-dye labeled antibodies against α-smooth muscle actin (α-SMA)-expressing cells). Reprinted from Gardenier JC, Hespe GE, Kataru RP, Savetsky IL, Torrisi JS, Nores GD, Dayan JJ, Chang D, Zampell J, Martínez-Corral I, Ortega S, Mehrara BJ. 2016. *JCI Insight* **1**(15):e84095, with permission.

cell, such as an immune cell or a target cell that the pathogen might affect. The altered animals can then be used in an experimental model system. For example, the role of a particular bacterial antigen in immunity can be interrogated by generating transgenic animals that express the antigen during an early developmental stage so that the animal does not immunologically react to the antigen upon exposure to the pathogen.

Humanized Transgenic Animal Models. The construction of transgenic mice that harbor human tissues or express specific human genes can provide a better model of human infection than normal mice. Immunodeficient transgenic or mutant mice, such as nude, SCID, NOG, or NSG mice (see Table 10-1), can be used as recipients for human genes, cells, or tissues because they do not react against the foreign material due to lack of an immune response. These "**humanized**" transgenic mice can then serve as infection models for determining the role of those immune components in human disease. For example, SCID mice, which lack a competent immune system, have been used as recipients for xenografts of human tissues or human stem cells. These SCID-human chimeric animals end up with an amazing complement of human immune cells, including T cells, B cells, natural killer cells, monocytes, macrophages, and dendritic cells, as well as organs and tissues with human cells in them, including liver, lung, and gastrointestinal (GI) tract. These chimeric animals serve as excellent *in vivo* models for infection studies and pre-clinical drug screening and toxicity studies.

In another example, "humanized" transgenic mice expressing the human enterocyte-associated protein E-cadherin receptor were used to study intestinal invasion by the foodborne intracellular pathogen *Listeria monocytogenes*. When *L. monocytogenes* is internalized inside a cell within an endosome, it normally escapes from the endosome using a membrane-disrupting toxin, called listeriolysin (LLO), which allows it to disseminate from cell to cell in the body (see chapter 11). *L. monocytogenes* has two surface proteins that act as adhesins: internalin A (InlA) that binds to E-cadherin and internalin B (InlB) that binds to hepatocyte growth factor receptor (Met). It was proposed that interaction of InlA with the E-cadherin receptor might allow *L. monocytogenes* to translocate across the gut epithelial layer directly, rather than having to invade through Peyer's patches. To determine the importance of InlA-E-cadherin-mediated translocation via enterocytes in invasive infections, "humanized" transgenic mice were colonized with either wild-type *L. monocytogenes* or mutants that did not produce InlA or InlB. These studies showed that InlA, and not InlB, is critical for invasion of the intestinal barrier following oral infection, but InlA is not important for infection when animals are infected intravenously. On the other hand, both InlA and InlB are important for crossing the blood-brain barrier to cause encephalitis and for crossing the placenta barrier to cause infection of the fetus during pregnancy.

Other Transgenic Animal Models. Transgenic animals harboring inducible reporter gene fusions in their genomes can be used to identify cells in which specific host responses have been activated during infection. For example, transgenic mice were constructed containing a transcription fusion between the bacterial *lacZ* (β-galactosidase) reporter gene (see chapter 9) and the control region of a mammalian gene regulated by NFκB, which is a transcription factor that regulates several genes involved in host immunity. This NFκB-responsive *lacZ* reporter was then used to monitor NFκB activation during infection with *L. monocytogenes* (Figure 10-2). Activation of NFκB was visualized by cleavage of chromogenic substrates by the β-galactosidase that was produced when the genes were turned on. During infection with wildtype bacteria, NFκB activation was visualized strongly in endothelial cells of the spleen, liver, and brain. However, this activation was not observed for a mutant *L. monocytogenes* strain lacking LLO, suggesting that this virulence factor is important for dissemination of the bacteria to these organs.

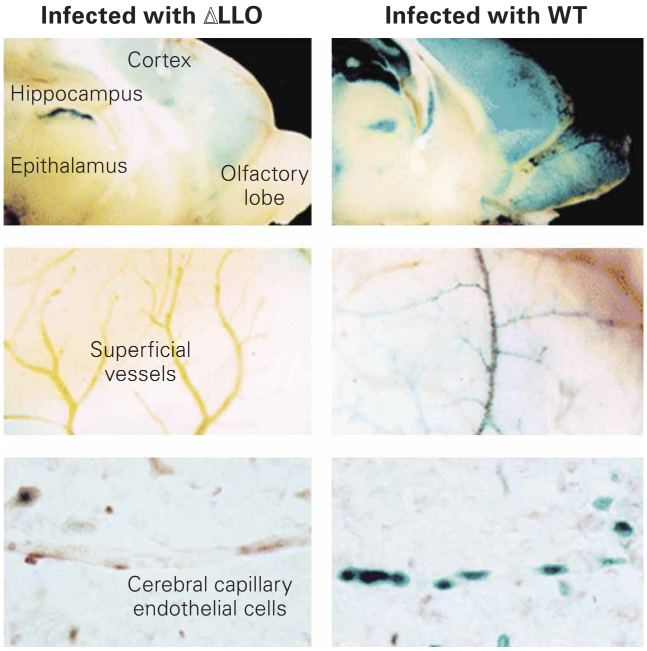


Figure 10-2. *In vivo* activation of NFκB in endothelial cells of transgenic mice during infection with *Listeria monocytogenes*. Transgenic mice containing an NFκB-dependent *lacZ* reporter gene were infected intravenously with wild-type *L. monocytogenes* or ΔLLO mutant bacteria. Brain slices were examined 48 hours after inoculation and stained for β-galactosidase activity (blue color). Compared to mice infected with mutant bacteria lacking LLO, mice infected with wild-type bacteria (WT) showed strong staining of the olfactory lobe, superficial blood vessels, and cerebral capillary endothelial cells lining the lumen of the vessels, indicating LLO-dependent activation of NFκB signaling in endothelial cells of the brain during infection. Adapted from Kayal S, Lilienbaum A, Poyart C, Memet S, Israel A, Berche P. 1999. *Mol Microbiol* **31:**1709-1722, with permission.

In vivo Imaging of Animals during Infection

Until recently, most host cell transcriptional and proteomic profiling studies have been performed in *in vitro* infection models using cell cultures. While these studies have provided unique and valuable insights into the complexity of host-microbe interactions during infection, researchers are now turning more and more toward ***in vivo* infection models** using **whole-body biophotonic imaging** technologies. These *in vivo* studies of gene expression, particularly coupled with transgenic animal models, are shedding new light on the dynamics of infection processes and disease progression.

The ultimate goal of infection biology is to study a pathogen in real time during infection and in its natural infectious environment, the living host. Whole-body biophotonic imaging, introduced in chapter 8, provides a noninvasive technique that allows researchers to do just that—study bacterial infections *in vivo*, including monitoring of disease profiles and conducting drug efficacy studies in live animals over the entire course of the disease. For this approach, the bacterial pathogen or the host animal (e.g., a transgenic mouse) is engineered with a reporter gene to express **bioluminescence** (visible light), either constitutively or upon induction during infection. The animal is anesthetized and the bioluminescence is monitored using ultra-sensitive, photon-counting video cameras that record images at extremely low light levels (Figure 10-3A and B). The bacterial bioluminescent reporter is often a constitutively expressed or an inducible **bacterial luciferase (*lux*)** operon that encodes all of the enzymes required to make the bacteria or host glow in the presence of ATP and oxygen (see chapter 8).

Sometimes other reporters of bacterial gene expression are used, such as **firefly (*Photinus pyralis*) luciferase (*luc*)**, which uses D-luciferin, oxygen, and ATP as substrates to produce oxyluciferin, AMP, inorganic pyrophosphate, water, and light (at 547 to 617 nm). In this case, the D-luciferin substrate needs to be injected into the animal before each reading. Fusions between bacterial proteins and fluorescent proteins have also been used as bacterial reporters in biophotonic imaging (see Figure 10-3). **Red fluorescent protein (RFP)**, instead of **green fluorescent protein (GFP)**, is often preferable for this application because animal bodies readily absorb more green light than red light.

Conversely, host genes can be fused to luciferase or fluorescent proteins to follow the expression of these host genes in different organs and tissues in response to encountering bacteria during infection. For example, in a transgenic mouse expressing a fusion of the IL-8 gene to insect luciferase (IL-8-Luc), the IL-8 gene is turned on when the host senses the presence of released bacterial products, such as lipopolysaccharide (LPS) or toxins through activation of signal transduction pathways (Figure 10-3C). In this case, the luciferin substrate was injected prior to injection of the cell-free supernatants from cultures of *Pseudomonas aeruginosa*.

Other methods of following infections by biophotonic imaging are being developed for pathogenic bacterial species or animal hosts that are not amenable to genetic manipulation. One approach is to tag molecules that bind specifically to bacterial surfaces, such as antibodies or lectins, with synthetic fluorescent dye labels. These tagged molecules can then be injected into the animals to locate and image the bacteria as the infection progresses. Combining the tagged bacteria with transgenic animals having reporter genes allows for dual monitoring of bacterial localization with host response.

Systems Genetics: Comparative Genomics of the Host Response

Pasteur recognized the importance of host factors, such as certain genetic traits, with regard to susceptibility to infection. Later, Charles Nicolle (1866-1936) noted the coexistence of asymptomatic and symptomatic infections in human populations. A number of genetic epidemiological studies conducted during the 1930s through the 1950s noted a number of inheritable genetic traits that were correlated with susceptibility to disease. A direct genetic link was first demonstrated in 1952 with immunodeficient children, where an X-linked recessive mutation in Bruton's tyrosine kinase (*BTK*) gene was shown to lead to agammaglobulinemia, a condition associated with severe infections due to deficiency in mature B cells and serum antibodies. This finding was followed in 1954 by the discovery of the sickle-cell anemia trait (a mutant allele in the hemoglobin β chain gene), which protected heterozygote individuals against malaria caused by infection with the parasite *Plasmodium falciparum*. Since then, many studies in animals and humans have shown a strong correlation of severity of infectious disease with genetically predisposed individuals having certain variant alleles in multiple genes.

Insights into the complex workings of the host, in the context of a large, highly diverse population, and its interactions with not only its environment, but also with its microbial inhabitants have largely stemmed from what is now termed the field of **systems genetics**—formerly termed **complex trait analysis (CTA)**, **genetical genomics** (combination of genetics with gene expression analysis), or **quantitative trait loci (QTL)** analysis (correlation of expression of DNA loci with variation in phenotype). Strategies used in systems genetics studies include making genetic and/or environmental perturbations in the experimental system and then following the effects on host transcription, translation, metabolite profiles, and phenotypes, which can also be coupled with microbiome profiles to build a correlation map at multiple interconnecting levels. Based on correlations of traits that occur among individuals within a population, researchers formulate biological interaction network models that can be tested in clinical trials.

Single Nucleotide Polymorphisms (SNPs). The most common type of genetic variation within a population occurs as a single base-pair change, referred to as a **single nucleotide**

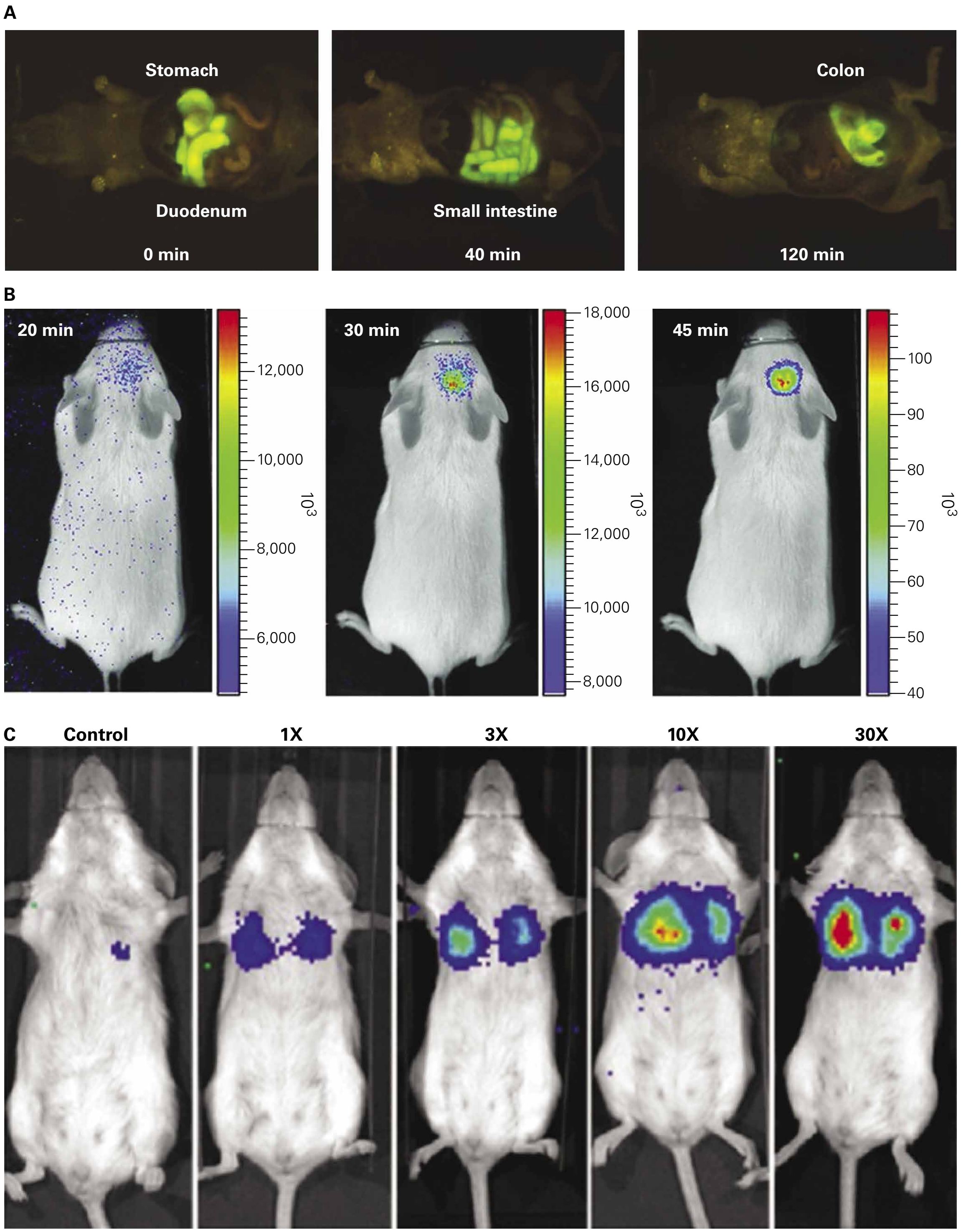


Figure 10-3. Noninvasive whole-body monitoring of bacterial infections using biophotonic imaging. (**A**) Imaging of GFP-expressing *E. coli* from outside intact infected animals to monitor the spatial-temporal flow of the bacteria through the GI tract. The green bacteria were introduced into the stomach via gavage, and images were taken over time to reveal the progression of the bacteria through the GI tract. Reprinted from Hoffman RM, Zhao M. 2006. *Nat Protoc* **1:**2988-2994, with permission. (**B**) Bioluminescence detected in mice inoculated in the skin over the head/neck area with *Bacillus anthracis* spores containing a plasmid with the *lux* reporter downstream of germination-specific promoters. The mice were monitored with an *in vivo* biophotonic imaging system to observe the bioluminescence emitted upon germination of the spores. Shown are the images 20, 30, and 45 minutes post injection. Reprinted from Sanz P, Teel LD, Alem F, Carvalho HM, Darnell SC, O'Brien AD. 2008. *Infect Immun* **76**(3)**:**1036-1047, with permission. **(C)** Noninvasive whole-body monitoring of lung inflammation induced by infection with cell-free *Pseudomonas aeruginosa* culture supernatants in IL-8-Luc transiently transgenic mice. Shown are images of mice transiently transgenized with IL-8-Luc, delivered to the mouse lungs by transfection of a reporter plasmid encoding the luciferase *luc* gene under control of a bovine promoter for IL-8. One week after transfection, the mice were injected intraperitoneally with luciferin substrate, followed by intratracheal exposure to increasing doses of *P. aeruginosa* cell-free supernatants. Reprinted from Stellari F, Bergamini G, Sandri A, Donofrio G, Sorio C, Ruscitti F, Villetti G, Assael BM, Melotti P, Lleo MM. 2015. *J Transl Med* **13**:251, with permission.

polymorphism (SNP; see chapter 5). SNPs can account for the majority of inter-individual genetic variations that affect host responses to pathogens. SNPs typically result in multiple alleles of genes, but most variants have minimal impact on biological function. The frequency of a SNP, particularly if it is determined to be associated with a disease outcome, is denoted by its occurrence as a less common allele in a population, relative to the minor allele frequency, which is the frequency at which the second most common allele occurs in the population.

To assist in the identification of SNPs associated with susceptibility or resistance to infection, a number of genetic reference populations of mice have been developed that can be used for genotype (SNP) mapping. Two such mouse populations that have proven powerful for conducting systems genetics are the **Collaborative Cross (CC)** and the **Diversity Outbred (DO)** heterogenous mouse stock collections that were derived from defined crossbreeding of a common set of eight founder mouse strains, comprised of three major *Mus musculus* subspecies: *M. m. musculus*, *M. m. domesticus*, and *M. m. castaneus* (Figure 10-4). These two populations have roughly 45 million SNPs, with up to eight unique alleles at any given gene (locus), and about 4 million indels (DNA insertion/deletions). Along with RNA-Seq technologies for transcript quantification of allelic expression and powerful bioinformatic software packages, the availability of the annotated whole-genome sequences of the eight founder strains has enabled the determination of haplotypes (i.e., DNA

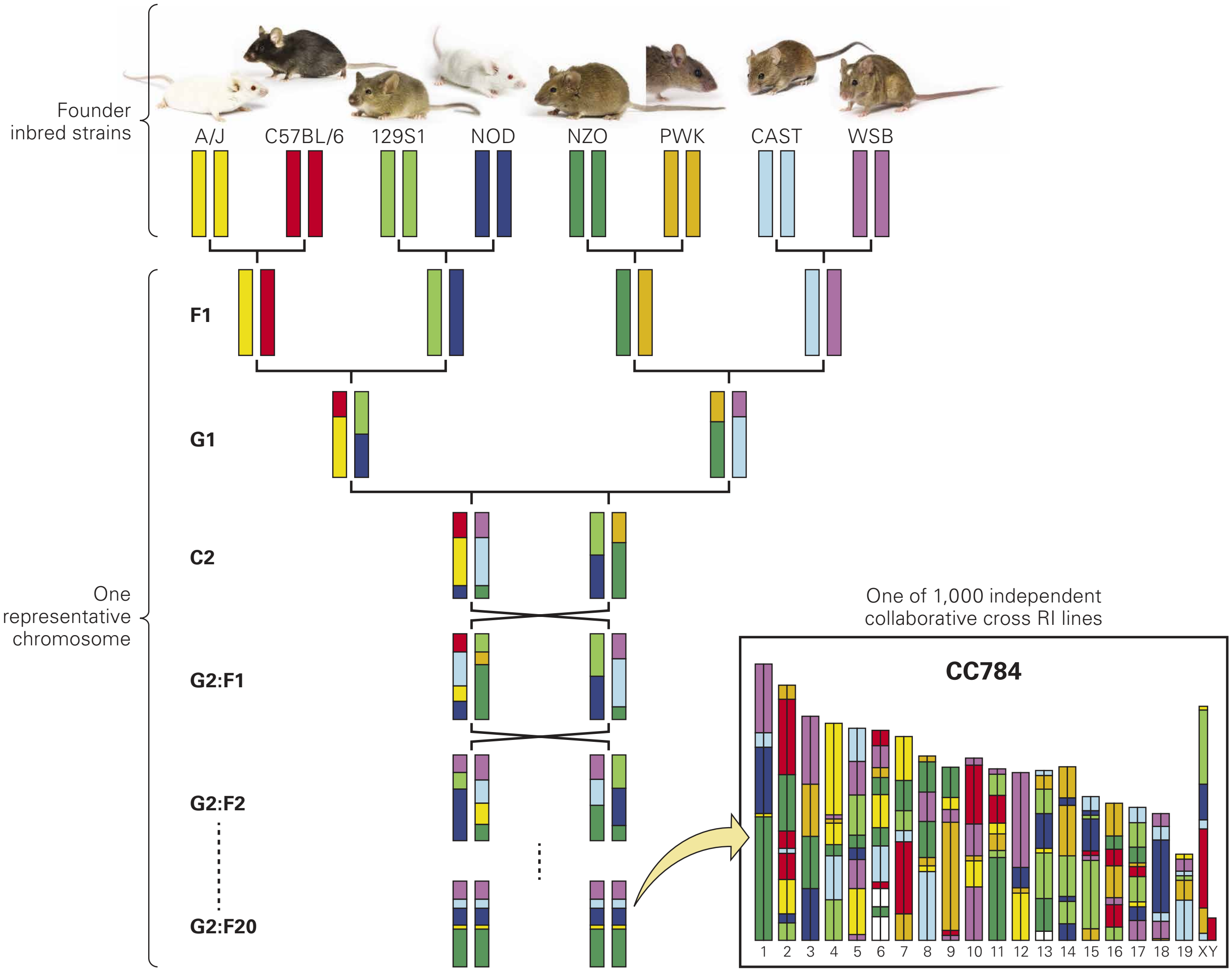

Founder inbred strains
A/J
C57BL/6
129S1
NOD
NZO
PWK
CAST
WSB
F1
G1
C2
One representative chromosome
G2:F1
G2:F2
G2:F20
One of 1,000 independent collaborative cross RI lines
CC784
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 XY

Figure 10-4. Defined crossbreeding of the eight parental mouse founder strains capture 90% of all genetic variation in Collaborative Cross (CC) mice. A well-planned crossbreeding of the eight parental mouse founder inbred strains (A/J, C57BL/6, 129S1, NOD, NZO, PWK, CAST, and WSB) through multiple generations and crosses resulted in recombinant inbred lines of mice that are a mosaic of the eight founder genomes. Shown is the breeding scheme, illustrated for one representative chromosome (chromosome 1), used to generate randomized variation in mouse line CC784, one of the 1,000 independent CC mouse recombinant inbred (RI) lines generated. Adapted from Nashef A, Agbaria M, Shusterman A, Lorè NI, Bragonzi A, Wiess E, Houri-Haddad Y, Iraqi FA. 2017. *Methods Mol Biol* **1488**:551-578, with permission.

variations inherited together) and genetic mapping of SNPs correlated with disease, as described in the next section.

Genome-Wide Association Studies (GWAS). One approach to identifying genes or genetic risk factors involved in host response to bacterial infection is to compare DNA sequence variations within the individual genomes of a population and then identify common variants (SNPs or indels) that are associated with increased or decreased risk of disease, as well as increased or decreased likelihood of responding well to treatments. The increased ability to perform whole-genome scans and SNP/indel analyses, including quantitative expression analyses, has enabled large-scale genotyping studies, commonly referred to as **genome-wide association studies (GWAS)** to map candidate genes with infectious disease outcome. GWAS can be performed with human populations as well as animals.

Within a population, different SNPs can have different degrees of **linkage**, such that an allele of one SNP could be inherited or correlated with an allele of another SNP. A **direct association** occurs when allelic changes in the SNP (or genotype) lead directly to a phenotypic trait, whereas an **indirect association** occurs when the allelic changes in the SNP does not directly lead to a phenotypic trait but occurs because it is near (or linked) with another SNP that does have a direct association with the trait. Since SNPs identified from GWAS could be indirectly associated with a disease trait due to the presence of a nearby SNP that has a dominant effect, additional studies must be conducted to refine the SNP locus that is directly associated with the observed phenotypic trait.

As an example, inbred mouse strains exhibit striking differences in the susceptibility of their macrophages to the effects of the lethal toxin produced by *Bacillus anthracis,* the causative agent of anthrax. By comparing two mouse strains with different reactivities, it was possible to show that this difference in susceptibility lies downstream of anthrax lethal toxin entry into macrophages. Subsequently, a locus controlling this phenotype, called *Ltxs1*, was mapped to chromosome 11, and the responsible gene was identified as a gene encoding Kif1C, a kinesin-like cellular motor protein. Multiple alleles of this gene were found to determine the susceptibility or resistance of mice to anthrax lethal toxin. Using a similar strategy, a novel genetic locus in BALB/cJ mice that confers resistance to *Yersinia pestis*, the causative agent of plague, was mapped to the major histocompatibility complex on chromosome 17.

Phenotypic traits that can be used as quantitative measures for assessing risk of disease (i.e., as **quantitative traits**) are ideal for identifying SNP/indel alleles (or genotypes) at well-defined genetic loci (QTLs) associated with the disease. These QTLs are consequently preferred for statistical analyses conducted in GWAS and are typically displayed as a **Manhattan plot** (Figure 10-5), which is a scatter plot with the genome sequence (as SNPs or indels) shown on the X-axis and the statistical significance of each SNP/indel allele being associated with the disease shown on the Y-axis as the negative logarithm of the association *P*-value. For example, a SNP that introduces a stop codon into the gene for Toll-like receptor 5 (TLR5), a protein in the membranes of host cells that controls the cellular response to bacterial flagella (see Figure 3-5), is directly associated with increased susceptibility to Legionnaires' disease caused by the flagellated Gram-negative bacterium, *Legionella pneumophila.* Likewise, two SNPs in another TLR, TLR4, which recognizes bacterial endotoxin

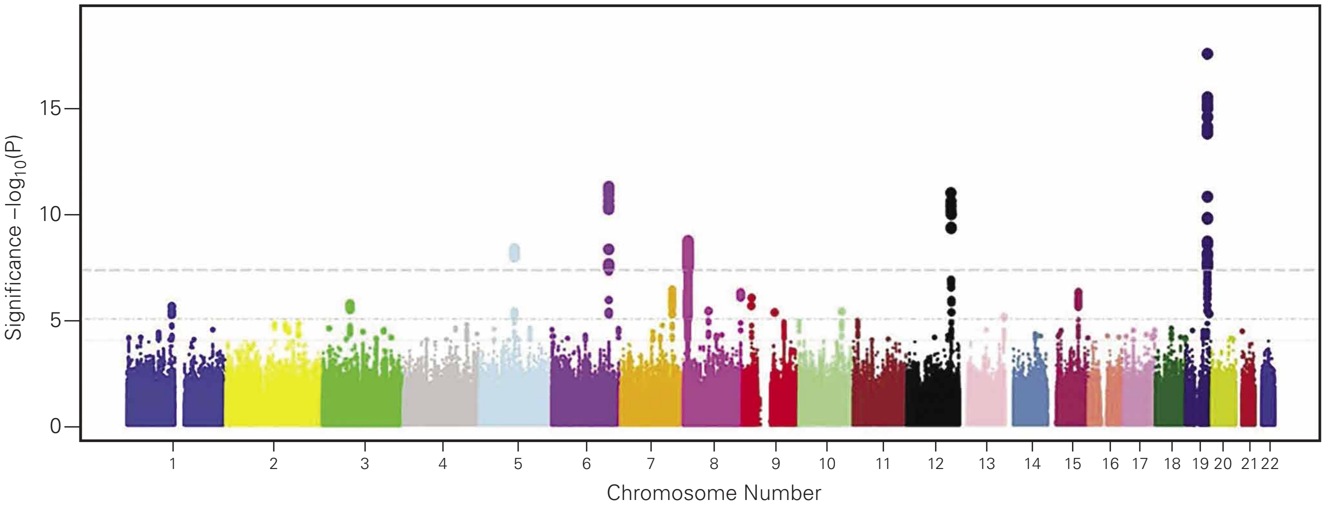


Figure 10-5. Manhattan plot used for GWAS to identify SNP associations with disease. A Manhattan plot is used to illustrate the genome-wide association of QTLs (or SNP loci) that are significantly associated with disease risk along the genome location of each chromosome (color coded) on the X-axis. SNP association with disease determined through GWAS is indicated as the negative logarithm of the statistical significance, *P*-value, corresponding to each SNP on the Y-axis. The strongest associations have the smallest *P*-values (e.g., *P*-value of 10^{-15}, which corresponds to a value of 15 along the Y axis). Data from Ikram MK, et al. 2010. *PLoS Genet* **6:**e1001184.

(LPS), are directly associated with increased susceptibility to infection with Gram-negative bacteria.

In addition to the single QTL analyses mentioned previously, GWAS also allows for **multilocus trait analysis (MLTA)** to identify interactions among different genetic variants within a genome. For example, the TLR4 and TLR5 SNPs mentioned previously are not directly associated with each other, but both are risk factors that when combined enhance the overall impact of each, and thereby amplify disease susceptibility. Indeed, the occurrence of both low-frequency alleles for TLR4 and TLR5 in an individual results in even greater increased risk or predisposition to severe disease upon infection with Gram-negative *L. pneumophila*.

It should also be noted that not all SNPs result in increased susceptibility to infection; some can result in decreased susceptibility. For example, a common SNP found frequently in individuals of European descent that is located within TLR1, which mediates host responses to a variety of bacteria, including mycobacteria, causes aberrant trafficking of TLR1 to the cell surface and thereby loss of TLR1 function. This so-called "loss-of-function" SNP, however, is associated with decreased incidence of leprosy, caused by *Mycobacterium leprae*.

Screening Approaches to Identify Host Factors Required for Infection

Genome-Wide Screening

Just as the availability of genomic sequences of bacterial pathogens has greatly increased our understanding of host-pathogen interactions, the whole genome sequences from the host species that they infect have also led to the identification of host factors contributing to bacterial virulence. And, importantly, next-generation genomics and analytical tools have enabled scientists to examine relationships between genetic and phenotypic variation on a scale that takes into account the population, using reliable quantitative measurements and robust statistical analyses. Key to all of these approaches is the thorough annotation of genomic sequences, and yet, as is the case with the bacterial genomes, there remains much to be completed. Comparative genomics among different hosts has been useful for filling in some of these gaps, particularly with regard to the underlying differences in host susceptibility to bacterial infections and the severity of disease. In recent years, two screening approaches have dramatically advanced the field: **gene silencing** (knocking down mRNA and protein levels) and **gene editing** (modifying or deleting genes and thereby their mRNA and protein products).

Gene Silencing via RNA Interference (RNAi). In 1998, it was discovered that exposing cells of the nematode *Caenorhabditis elegans* to gene-specific double-stranded RNA (dsRNA) resulted in specific and efficient gene silencing that was more potent than using sense or anti-sense RNA. This new gene-silencing technology, called **RNA interference (RNAi)**, enabled the screening of large libraries of host genes from any eukaryotic organism to identify host genes required for bacterial pathogenesis, particularly those for intracellular pathogens where host machinery play important roles in survival and propagation of the pathogen.

In RNAi screening, a synthetic dsRNA molecule (usually 21-23 nucleotides long), called **short interfering RNA (siRNA)**, is introduced through **transfection** into mammalian cells. Its presence inside the cell induces selective cell-mediated degradation of any mRNA that has homology to the siRNA, resulting in post-transcriptional gene silencing (Figure 10-6). A major advantage of RNAi-library screening technology is the ability to scale it up for genome-wide, high-throughput screening of thousands of genes simultaneously. To overcome problems with poor transfection efficiencies of synthetic or plasmid vector-derived siRNA, several viral-based vectors have been developed to increase delivery of the siRNA into mammalian cells. One challenge encountered with large-scale, RNAi-based screening approaches is overcoming poor reproducibility between screens due to off-target effects that interfere with identifying statistically significant differences from single screen runs. Multiple parallel screens are usually performed using the same RNAi libraries to improve the statistical power for detection of potential hits that are unique for individual pathogens as well as shared among different pathogens.

One of the first applications of RNAi technology was toward identifying host factors required for infection by the intracellular pathogen *L. monocytogenes*. A large dsRNA library was constructed, in which RNAi targeted nearly all of the **open reading frames (ORFs)** of the *Drosophila* genome for silencing. This valuable resource was then used to develop a fluorescence microscopy-based, high-throughput RNAi screening assay, in which particular cellular phenotypes associated with specific defects in the infection process were identified (Figure 10-7). In this screen, *Drosophila* cells were treated with dsRNA for about 4 days to down-regulate (silence) the target genes. This was then followed by infection for 24 hours with *L. monocytogenes* that was expressing GFP as a fluorescent marker for visualization of the bacteria. The mammalian cells were then fixed, and the host nuclei were stained with blue Hoechst dye. An automated fluorescence microscopy imaging system was set up to record the images of green bacteria with blue host nuclei.

Normally *L. monocytogenes* invades a host cell and escapes the vacuole in which it was internalized. Then, the bacteria replicate inside the invaded host cell and subsequently move to adjacent cells. Accordingly, images were discriminated based on five different phenotypes observed: (1) normal phenotype (control cells with no dsRNA treatment), where host cells have bacteria mostly inside the

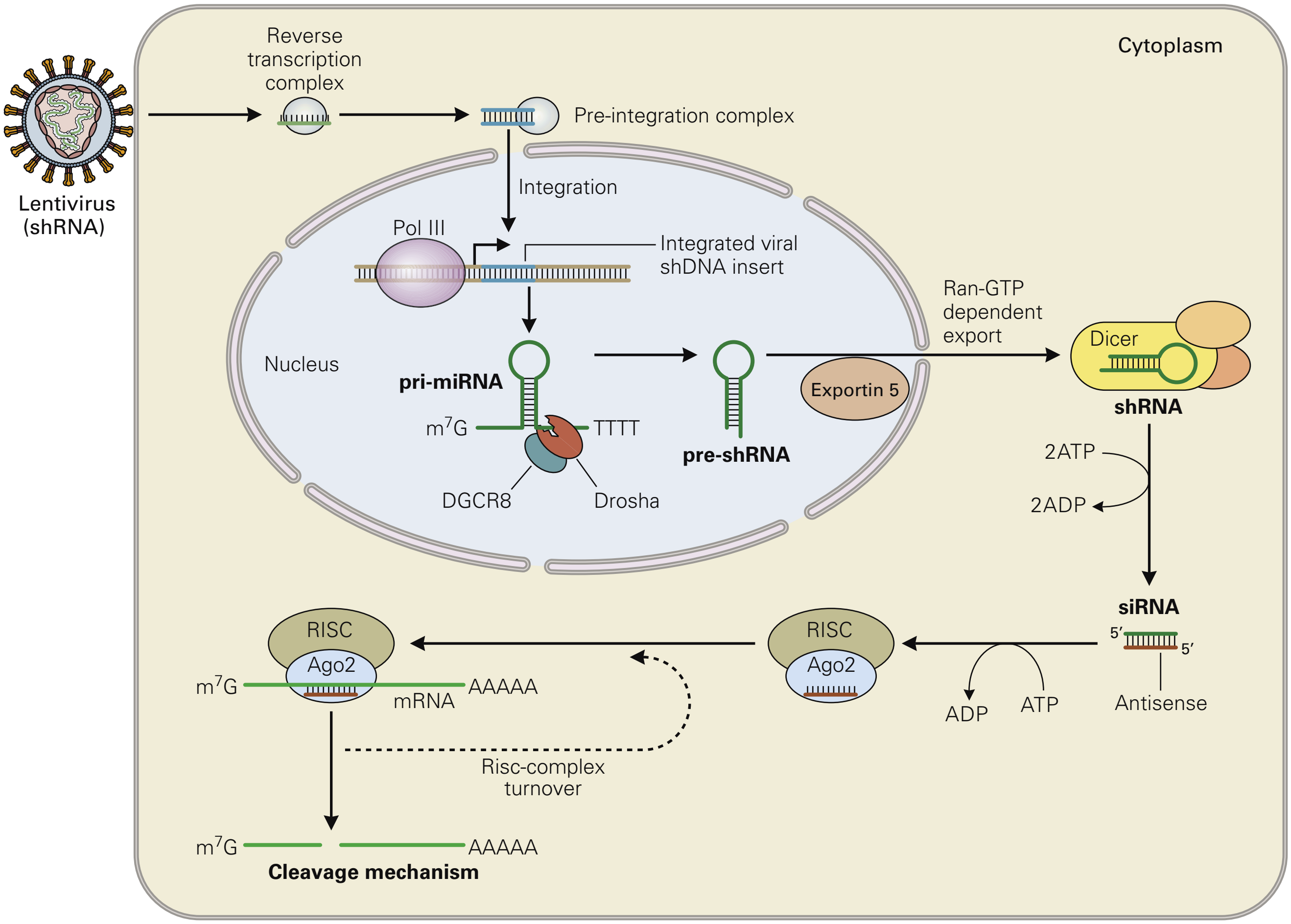

Reverse transcription complex
Cytoplasm
Pre-integration complex
Lentivirus (shRNA)
Integration
Pol III
Integrated viral shDNA insert
Ran-GTP dependent export
Nucleus
pri-miRNA
Dicer
Exportin 5
shRNA
m^{7}G
TTTT
pre-shRNA
2ATP
2ADP
DGCR8
Drosha
siRNA
RISC
Ago2
RISC
Ago2
5′
5′
m^{7}G
AAAAA
mRNA
ADP
ATP
Antisense
Risc-complex turnover
m^{7}G
AAAAA
Cleavage mechanism

Figure 10-6. Posttranscriptional gene silencing in mammalian cells by RNAi. In mammalian cells, RNAi occurs through the action of the enzyme Dicer, which exhibits RNase III-like activity, and the RNA-induced silencing complex (RISC), which is a group of cytoplasmic proteins with endonuclease activity that degrades the target mRNA. Dicer processes double-stranded RNA (dsRNA) first into short-interfering RNA (siRNA) of 20-25 base pairs, and a single strand of the siRNA (the guide strand) is incorporated in the RISC. This guide strand (shown in red) then base pairs with the complementary sequence on the target mRNA and induces cleavage of the mRNA by Argonaute 2 (Ago2), the catalytic component of the RISC, thereby silencing the gene expression such that no protein is made. In RNAi technology, this process can be mimicked by introduction of synthetic siRNA or transfection with plasmid or viral vectors (such as *Lentivirus* shown here) that encode a 70-nucleotide short hairpin RNA (shRNA) and are designed to produce siRNA through use of RNA polymerase III-dependent promoters to generate a primary microRNA transcript (pri-miRNA). The pri-miRNA transcript is then processed by a complex of RNase-III enzyme (called Drosha) and a dsRNA-binding protein (called DGCR8) to a pre-microRNA (pre-miRNA) with a characteristic stem-loop structure (pre-shRNA). The pre-shRNA is transported by Exportin 5 from the nucleus to the cytoplasm, where it is further processed by Dicer and RISC.

cytosol; (2) absent phenotype, where there are no bacteria inside the host cells, presumably due to knockdown of a host factor needed for adhesion or invasion; (3) decreased internalization phenotype, where only a few bacteria are found inside the host cells, presumably due to knockdown of a host factor that allows bacteria to escape from the vacuole and/or to replicate in the vacuole or the cytosol; (4) spots phenotype, where bacteria are clustered into spots or loci within the host cell cytosol, presumably due to knockdown of a host factor needed for escape from the entry vacuole, but the bacteria still can replicate within the vacuole; and (5) up phenotype, where there is an increased number of bacteria found inside the cell cytosol compared to normal phenotype, presumably due to knockdown of a host factor that allows for cell-to-cell spread (via actin-based motility) such that the bacteria accumulate inside the cytosol of the first invaded cell. The putative host factors required for *L. monocytogenes* infection identified through this screen spanned a wide range of cellular functions, including host proteins involved in cell adhesion, endocytic and vesicular trafficking, signal transduction, and cytoskeletal organization.

Initially, gene-silencing studies were performed in lower eukaryotes, such as nematode worms (*C. elegans*) or fruit flies (*Drosophila melanogaster*), but now this technology can be applied to most mammalian systems, including humans. Indeed, RNAi technology has been adapted for high-throughput, genome-wide screening of genes from any

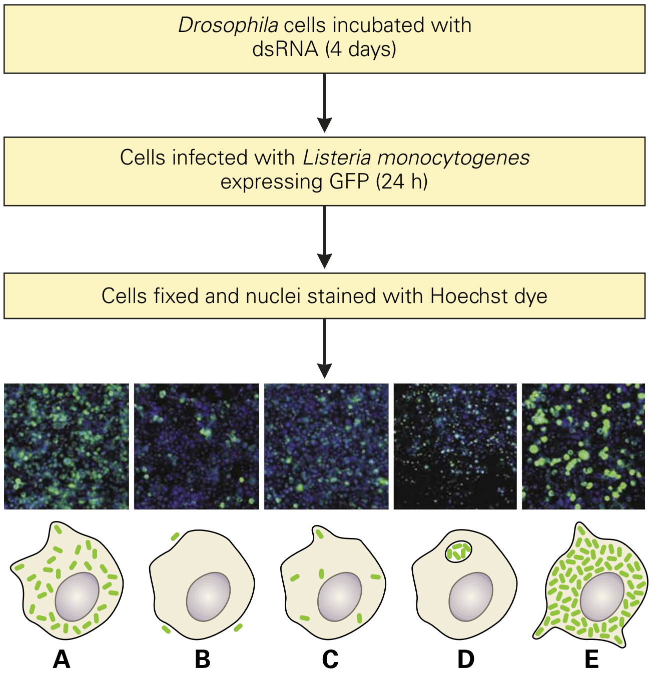

Drosophila cells incubated with dsRNA (4 days)
Cells infected with *Listeria monocytogenes* expressing GFP (24 h)
Cells fixed and nuclei stained with Hoechst dye
A
B
C
D
E

Figure 10-7. Genome-wide RNAi screening procedure for identifying host factors involved in *Listeria monocytogenes* pathogenesis and phenotypes observed. *Drosophila* S2 cells were incubated with dsRNAs for 4 days to allow for inhibition of host protein expression. The cells were then infected with the bacteria, followed by microscopy and image analysis of the infection phenotypes. Shown are representative micrograph images of each of the observed phenotypes: **(A)** control (no dsRNA); **(B)** absent phenotype with defect in adhesion or invasion; **(C)** decreased internalization phenotype with few cells inside; **(D)** spots phenotype with clustering of bacteria inside cellular vacuoles; and **(E)** up phenotype with increased number of bacteria accumulating inside cells. Below each image is a schematic depiction of the corresponding phenotype. Adapted from Agaisse H, Burrack LS, Philips JA, Rubin EJ, Perrimon N, Higgins DE. 2005. *Science* **309**(5738):1248-1251, with permission.

organism for which an annotated genome sequence is available and a bacterial infection model has been developed. As more host factors involved in bacterial pathogenesis are identified for different bacteria and different hosts, comparative analyses will undoubtedly reveal common pathogenic mechanisms used by different bacteria, as well as mechanisms unique for any single pathogen or disease.

Because bacteria do not have the mammalian machinery needed for the RNAi effect, siRNA specifically inhibits only the host gene products that are expressed during infection. This makes RNAi technology ideal for identifying potential host targets for therapy, and results from these studies could provide insights into new therapeutic strategies. For example, one study showed that RNAi could be applied *in vivo* in adult mice to delay the onset of LPS-induced sepsis by administration of liposomes containing siRNA that inhibited expression of TNF-α, an inflammatory cytokine normally turned on during sepsis.

Gene Editing via CRISPR-Cas9. Over the last few years a major revolution has transformed our ability to rapidly and precisely manipulate host genomes to study gene function and the role of host factors in disease progression *in vivo* in all kinds of model organisms, including primates. Three different genome-editing technologies have emerged that enabled introduction of mutations into targeted sites within host genomes: plant-derived transcriptional activator-like effector nucleases (**TALENs**), engineered zinc-finger nucleases (**ZFNs**), and bacterial clustered regularly interspaced short palindromic repeat (**CRISPR**) sequences plus CRISPR-associated (**Cas**) proteins (see chapter 7), specifically the **CRISPR-Cas9** system.

The ZFN and TALEN approaches use DNA-binding domains to guide the nuclease to the specific DNA site of cleavage, whereas the CRISPR-Cas9 system uses RNA to guide the nuclease to the complementary DNA sequence where cleavage then occurs. In each of these genome-editing technologies, the "programmable" nuclease (i.e., the nuclease that can be engineered or directed to bind and cleave a particular target sequence) induces double-stranded breaks at specific DNA sequences (Figure 10-8). The host then repairs these breaks by either a **homology-directed repair (HDR)** mechanism if homologous sequences are available to use as a template, which leads to precise gene correction or replacement, or by a **nonhomologous end joining (NHEJ)** mechanism, which may introduce small insertions or deletions (indels) that result in frameshift mutations or sometimes genome rearrangements.

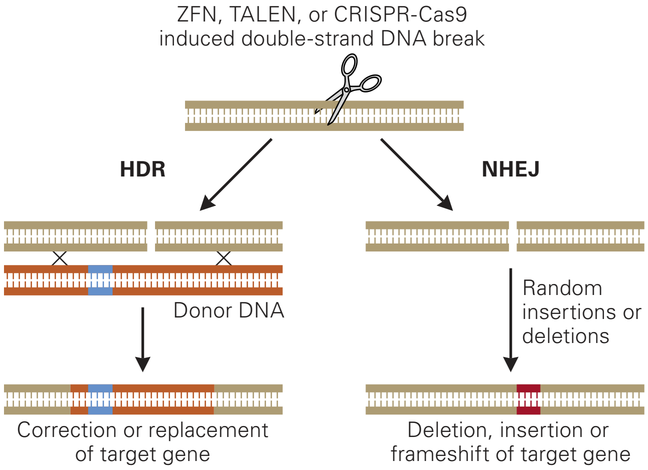

ZFN, TALEN, or CRISPR-Cas9 induced double-strand DNA break
HDR
NHEJ
Donor DNA
Random insertions or deletions
Correction or replacement of target gene
Deletion, insertion or frameshift of target gene

Figure 10-8. Genome-editing technologies rely on host DNA repair mechanisms to introduce mutations. Following DNA double-strand breaks introduced by ZFN-, TALEN-, or CRISPR-Cas9-based genome-editing technologies, the DNA can be repaired by either homology-directed repair (HDR), if a donor template is available, or by nonhomologous end joining (NHEJ) repair. HDR can lead to correction or replacement of the target gene, while NHEJ can lead to random insertions or deletions (indels) that result in frameshift mutations or sometimes genome rearrangements.

The ZFN and TALEN approaches suffer from technical difficulties in engineering, expressing and purifying the large bulky proteins containing multiple guiding domains coupled to their nuclease domains, and so they have not advanced very rapidly in the field. So, while they may have some advantages, such as greater specificity and fewer off-site target effects, we will not discuss these approaches any further here, and instead we direct the interested reader to selected citations at the end of this chapter. In contrast, the CRISPR-Cas9 system is much easier to manipulate and far more versatile. As such, it has skyrocketed in the last few years as the leading genome-editing technology used for nearly all applications.

The CRISPR-Cas9 system most frequently used is a genetically engineered version of the one from *Streptococcus pyogenes* (Figure 10-9), where site-specific DNA cleavage by the Cas9 nuclease is directed by a complementary sequence on a **single guide RNA (sgRNA)**, which is a synthetic chimeric RNA derived from fusion of a CRISPR RNA (**crRNA**) with the trans-activating crRNA (**tracrRNA)**. The crRNA has a sequence of about 20 nucleotides at the 5' end that is complementary to the target DNA recognized immediately downstream of the **protospacer-adjacent motif (PAM)** that is located on the opposite DNA strand and a short sequence at the 3' end that is complementary to a portion of the tracrRNA, which serves as an adaptor between Cas9 and the crRNA. Recognition and unwinding of the dsDNA around the PAM sequence, which for *S. pyogenes* Cas9 is NGG (or sometimes NAG), induces double-strand DNA cleavage by two different nuclease domains in the Cas9 protein: the **RuvC** domain nicks the DNA strand that has the PAM motif and the **HNH** domain nicks the DNA strand that is bound to the guide crRNA.

The power of CRISPR-Cas9 technology as a genome-wide loss-of-function screening tool was recently demonstrated by a number of studies seeking to identify host factors required for host susceptibility to infection or for mediating cytotoxicity due to infection. One such study used the CRISPR-Cas9 system to identify host proteins necessary for α-hemolysin-mediated toxicity during infection with *Staphylococcus aureus*. Previous studies had implicated that the cytotoxic effects of the toxin on myeloid cells was important for the severity of infection outcome. To identify host factors that mediated this toxin-induced effect, the researchers transduced the human myeloid cell line U937 with a CRISPR-Cas9 lentiviral library encoding about 120,000 sgRNAs targeting over 19,000 human genes with six sgRNAs per gene and 1,864 microRNAs (miRNAs) with four sgRNAs each. The resulting library of myeloid cells with gene knockdowns were treated with α-hemolysin, and the cytotoxicity was monitored by flow cytometry using a membrane-impermeable dye to screen for cells with damaged membranes that had taken up the dye. Cells transduced with lentivirus encoding sgRNAs that were resistant to toxin treatment were selected, and the corresponding sgRNAs were sequenced to identify the genes that were targeted. As expected, among the 10 genes knocked down in cells enriched after intoxication was the known cell surface receptor for α-hemolysin, called ADAM10, but also found was a protein involved in regulating the display of ADAM10 on the cell

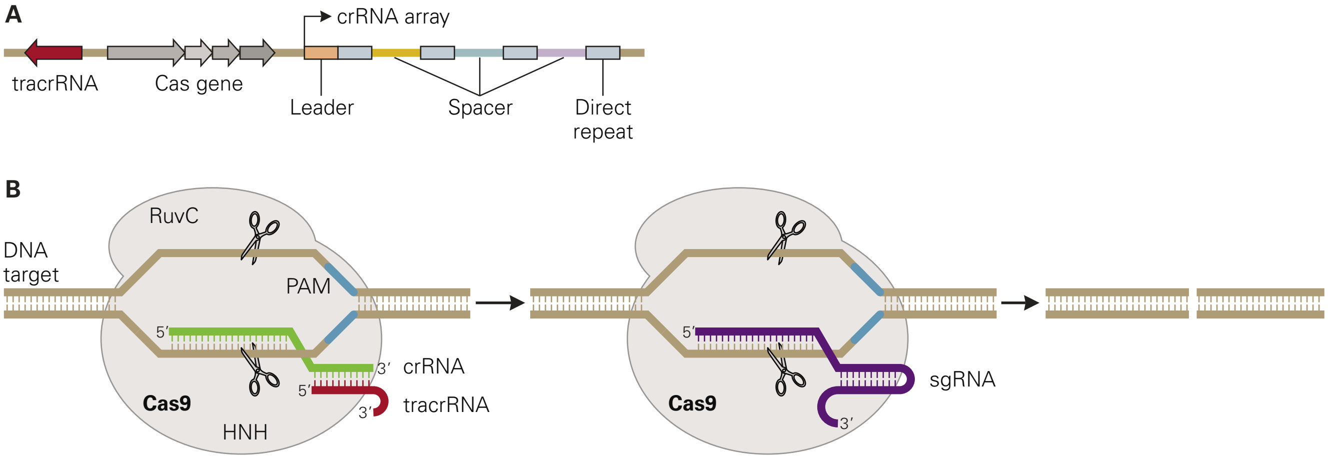


Figure 10-9. CRISPR-Cas9-mediated genome editing. (A) Schematic of the CRISPR-Cas locus from *Streptococcus pyogenes*. Each crRNA gene is comprised of a conserved direct repeat DNA plus a spacer DNA that corresponds to an incorporated piece of foreign DNA. **(B)** Schematic depicting Cas9 bound to target DNA. Cas9 is comprised of two DNA nuclease domains, RuvC and HNH. The HNH domain of Cas9 nicks the target DNA strand that is bound to the guide RNA, and the RuvC domain of Cas9 nicks the opposite strand. Site-specific cleavage by Cas9 nuclease is directed by complementary binding of the target DNA sequence directly downstream of the protospacer-adjacent motif (PAM) sequence (NGG) on the opposite strand and an engineered single guide RNA (sgRNA) (right panel), derived from fusion of the tracrRNA and the crRNA with a spacer portion that is complementary to the target DNA (left panel). The double-stranded DNA break is repaired by HDR or NHEJ, as depicted in Figure 10-8.

surface. Three other identified proteins included host proteins known to be involved in cellular uptake and trafficking pathways used by other bacterial toxins: sphingomyelin synthase 1 (SGMS1), a regulator of sphingomyelin-enriched lipid raft formation; SYS1, a protein involved in Golgi trafficking; and ADP-ribosylation factor 1 (ARF1), a small GTPase found in the Golgi that regulates vesicular trafficking.

In another recent study, genome-wide CRISPR-Cas9 screening was used to identify host factors conferring susceptibility to bacterial type 3 secretion system (T3SS)-mediated cytotoxicity during infection with *Vibrio parahaemolyticus*. As shall be explored further in chapter 13, T3SSs are present in many pathogens and are specialized secretion systems that can deliver secreted toxic effector proteins directly into host cells during infection to cause various cytotoxic effects (see Figure 13-7). *V. parahaemolyticus* possesses two such secretion systems, T3SS1 and T3SS2. Mutant bacteria producing only one of these systems still cause rapid cell death in the human intestinal epithelial cell line HT-29, but with different kinetics. To identify the host factors affected by each of the secretion systems, the HT-29 cells were transduced with a CRISPR-Cas9 lentiviral library encoding nearly 75,000 sgRNAs with four sgRNAs per gene. The resulting library of cells knocked down for each of the targeted genes was then infected with *V. parahaemolyticus* producing either T3SS1 or T3SS2. Any surviving cells were considered resistant to bacterial killing by the respective T3SS due to decreased expression of a particular sgRNA-targeted host protein that was important for susceptibility or cytotoxicity mediated by the T3SS. The integrated sgRNAs from surviving cells were then sequenced to determine which genotypes were most enriched in each of the screening experiments. Results indicated that distinct host mechanisms mediate susceptibility to infection through each of the secretion systems (Figure 10-10).

T3SS1 resistance was primarily associated with enrichment of sgRNAs targeting multiple genes important for cell surface sulfation, the SWI/SNF chromatin remodeling

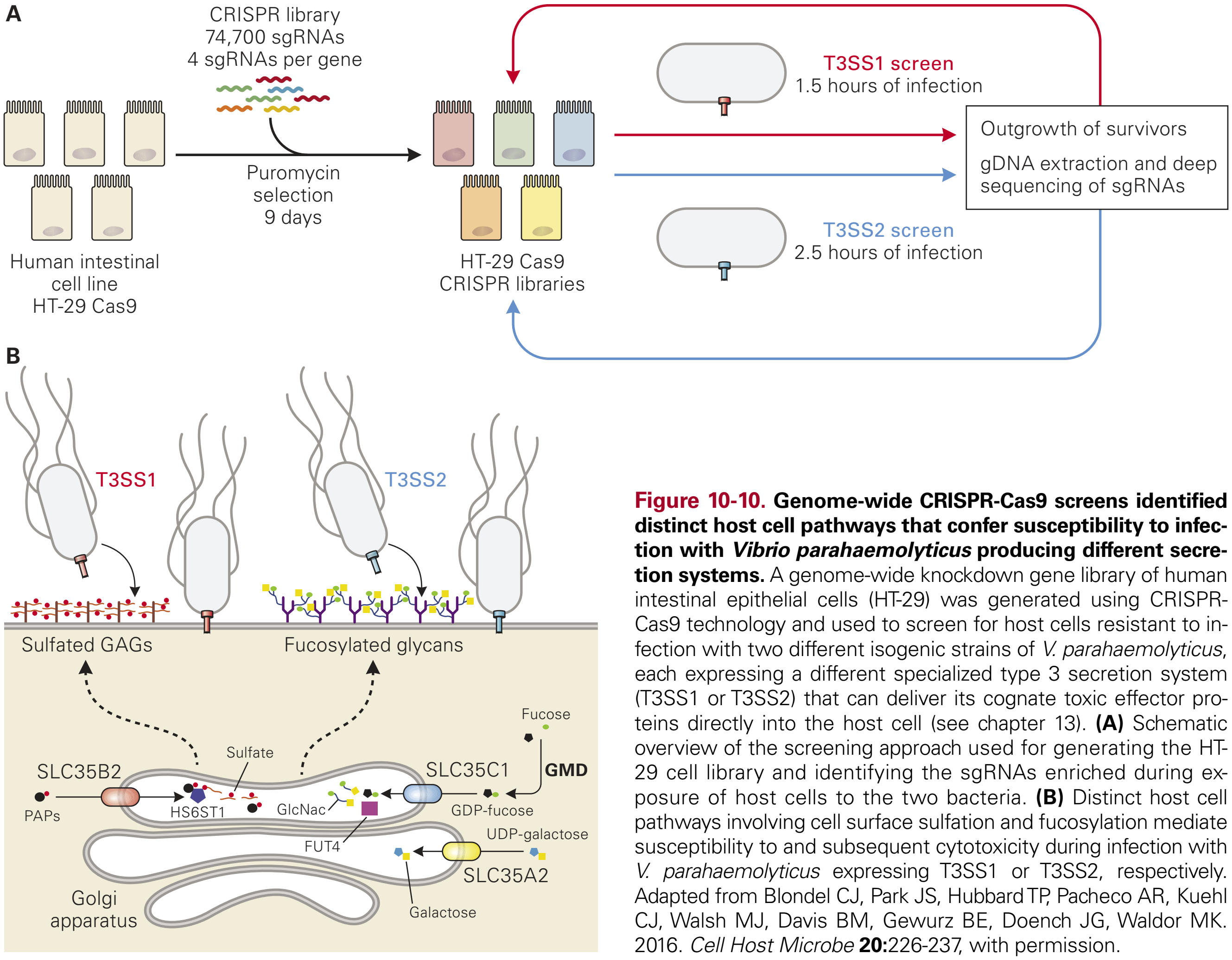

A
CRISPR library
74,700 sgRNAs
4 sgRNAs per gene
Puromycin
selection
9 days
Human intestinal
cell line
HT-29 Cas9
HT-29 Cas9
CRISPR libraries
T3SS1 screen
1.5 hours of infection
T3SS2 screen
2.5 hours of infection
Outgrowth of survivors
gDNA extraction and deep
sequencing of sgRNAs
B
T3SS1
T3SS2
Sulfated GAGs
Fucosylated glycans
Fucose
GMD
Sulfate
SLC35B2
SLC35C1
PAPs
HS6ST1
GlcNac
GDP-fucose
FUT4
UDP-galactose
SLC35A2
Golgi
apparatus
Galactose

Figure 10-10. Genome-wide CRISPR-Cas9 screens identified distinct host cell pathways that confer susceptibility to infection with *Vibrio parahaemolyticus* producing different secretion systems. A genome-wide knockdown gene library of human intestinal epithelial cells (HT-29) was generated using CRISPR-Cas9 technology and used to screen for host cells resistant to infection with two different isogenic strains of *V. parahaemolyticus*, each expressing a different specialized type 3 secretion system (T3SS1 or T3SS2) that can deliver its cognate toxic effector proteins directly into the host cell (see chapter 13). **(A)** Schematic overview of the screening approach used for generating the HT-29 cell library and identifying the sgRNAs enriched during exposure of host cells to the two bacteria. **(B)** Distinct host cell pathways involving cell surface sulfation and fucosylation mediate susceptibility to and subsequent cytotoxicity during infection with *V. parahaemolyticus* expressing T3SS1 or T3SS2, respectively. Adapted from Blondel CJ, Park JS, Hubbard TP, Pacheco AR, Kuehl CJ, Walsh MJ, Davis BM, Gewurz BE, Doench JG, Waldor MK. 2016. *Cell Host Microbe* **20:**226-237, with permission.

complex, and collagen biosynthesis. Further experiments using cell-based assays confirmed that T3SS1-mediated proteoglycan sulfation increases bacterial adhesion and subsequent cytotoxicity. In contrast, T3SS2 resistance was associated with enrichment of sgRNAs targeting genes involved in fucosylation of exported cell surface glucans, protein Ser/Thr kinases, and the cytoskeletal protein spectrin. Additional experiments confirmed that T3SS2-mediated glucan fucosylation enhanced insertion of the T3SS2 translocon (tip of the needle apparatus) into host cell membranes for increased delivery of the toxic effectors into the host cells.

Host Response Profiling to Identify Host Factors Required for Infection

Profiling of host transcriptional, proteomic, and metabolomic responses to infection can be used to identify host genes, proteins, peptides, and metabolites involved in pathogenicity and monitor host responses to colonization and infection by bacteria on a global scale. For example, numerous innate immune factors are consistently up- or downregulated in host cells infected with pathogenic bacteria compared to cells without exposure to any microbe. Even in germ-free mice, reconstitution with commensal bacteria has been shown to upregulate expression of colonic epithelial cell genes associated with growth and innate immune responses.

Transcriptomics

The **transcriptional response (transcriptome)** of the host can be specific and distinct for the particular microbial challenge, whether commensal, pathogen, or a complex microbial community (see chapter 5). **Microarray technology** and **Illumina RNA-Seq technology** provide a profile of the expression patterns of thousands of genes (**transcriptomics**) in parallel in a single experiment and can answer questions about which genes are turned on or off under one condition compared to another condition as well as over time. There is also growing evidence that the host transcriptome response to microbial communities may influence the phenotypic outcome in a way that is different from the influence of individual microbes alone and could explain certain discrepancies observed between some clinical manifestations and those observed in laboratory infection models.

Microarray and RNA-Seq Technologies. The utility of using **microarrays** to identify and monitor genes differentially regulated by certain immune cytokines was first demonstrated in 1998 for the regulation of genes in human cells that respond to treatment with interferons (IFN-α, IFN-β, and IFN-γ). Within a short period of time thereafter, a burst of reports appeared in the scientific literature, exploiting this strategy for observing inflammatory cytokine and chemokine regulation, as well as apoptotic and other signaling pathways. By 2000, microarrays were gaining considerable popularity for profiling the host response to bacterial pathogens, such as *S. enterica*, where it was shown that not only the live bacteria, but also certain bacterial factors, such as LPS, could significantly impact the host transcriptome.

The contributions of specific virulence factors to the host response and vice versa can be characterized by comparing changes in host gene expression profiles following adherence of bacteria to host cells (for an example, see Figure 10-11) or

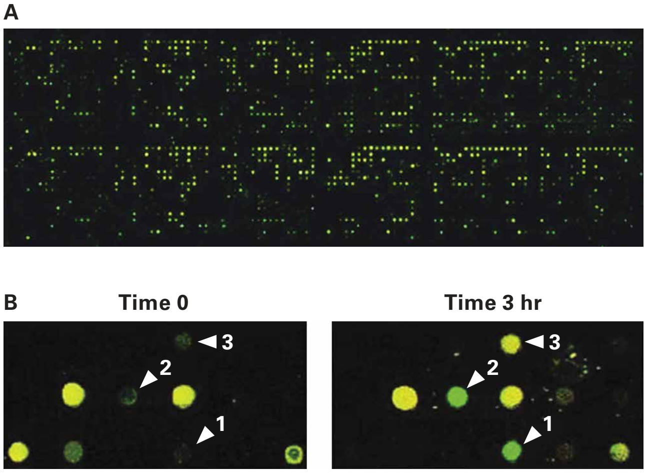


Figure 10-11. Identification of differentially regulated host genes by microarray expression profiling of human epithelial cells upon adherence of *Pseudomonas aeruginosa*. (A) Shown is an image of a microarray used to analyze mRNA transcript levels in cultured lung cells after incubation with *P. aeruginosa*. Total RNA was extracted from the cells at time 0 and after 3 hours incubation with *P. aeruginosa*. Each RNA sample was used as a template for reverse transcriptase synthesis of cDNA probes, which were incorporated with either Cy3- or Cy5-dCTP. The sample at time 0 was labeled with Cy3-dCTP (a green dye) and the sample after 3 hours incubation was labeled with Cy5-dCTP (a red dye). The probes were mixed and hybridized to a microarray slide, and the slide was scanned in a dual-laser scanning confocal microscope. **(B)** Shown is an enlargement of a region of the microarray showing adherence-dependent up-regulation of three genes during a time course of infection (at time 0 and 3 hours) with wild-type *P. aeruginosa* strain PAK (green) and a pilin deletion mutant of *P. aeruginosa* strain PAK-NP (red): the transcription factor interferon regulatory factor 1 (IRF-1) that regulates expression of IFN-responsive genes (arrow 1), the mitogenic transcriptional regulator c-Jun (arrow 2), and tissue factor pathway inhibitor 2 (TFPI2), a serine protease inhibitor involved in extracellular matrix remodeling (arrow 3). Yellow spots indicate a mixture of the two dyes, meaning that those host genes were expressed in cells exposed to wild-type or mutant bacteria. For example, whereas exposure to either bacteria induced expression of TFPI2, only wild-type bacteria with pili induced expression of IRF-1 and c-Jun. Adapted from Ichikawa JK, Norris A, Bangera MG, Geiss GK, van't Wout AB, Bumgarner RE, Lory S. 2000. *Proc Natl Acad Sci USA* **97**(17):9659-9664, with permission.

following infection with wild-type or mutant bacteria. For example, individual or combined effects of host gene expression were examined in response to infection with *P. aeruginosa* strains producing one or more toxic effector proteins, ExoS, ExoT, and/or ExoU (see chapter 12), which are injected directly into host cells by a specialized T3SS (see chapter 13). The host responses to these proteins revealed transcriptome patterns consistent with the suspected functions of these bacterial effector proteins.

Similarly, the virulence roles of various toxic effector proteins (called Yops for *Yersinia* outer proteins) secreted by the T3SS of *Yersinia enterocolitica* were delineated by monitoring the concerted actions of the different Yop effectors on the expression of host genes. Using mutants of *Y. enterocolitica* with deletions in genes that encoded two different Yop proteins, YopP and YopM, it was possible to determine from the types of genes regulated by these proteins that the primary function of the YopP effector was to counteract the host cell pro-inflammatory responses. At the time, it was not known what the function of YopM was, but based on the microarray analysis, it was determined that YopM induced regulation of genes involved in control of the cell cycle and cell growth, hinting for the first time at a possible role for YopM. This was later confirmed when YopM was indeed localized to the nucleus of host cells (see chapter 13).

The main challenges with microarray technology include the difficulties with normalization of replicates within and between experiments; universal agreement on what level of gene expression changes signifies “real” differences; and how to organize, interpret, and extrapolate the enormous amounts of data obtained from even a single experiment into useful information about biological processes that can be translated into new targets for disease diagnosis, intervention, and treatment. One recommendation from those who have used microarrays extensively is to use **quantitative real-time polymerase chain reaction (qPCR**; see Figure 5-5) to verify interesting differences rather than rely on microarray analysis alone.

A superior recent alternative method for quantifying gene expression profiles is **RNA-Seq technology**. In fact, the Illumina RNA-Seq platform now appears to be the preferred method for genome-wide transcriptomic analysis of all expressed genes in individual mammalian cells or in tissues under multiple different conditions or over time (Figure 10-12). An important step often performed in RNA-Seq methods for eukaryotic organisms that is not done for bacteria (see Figure 5-12) is the use of poly-A tail selection to capture mature, completely processed mRNA transcripts, which facilitates discrimination of mRNA from other RNAs and simplifies subsequent bioinformatic analysis and quantification of transcripts.

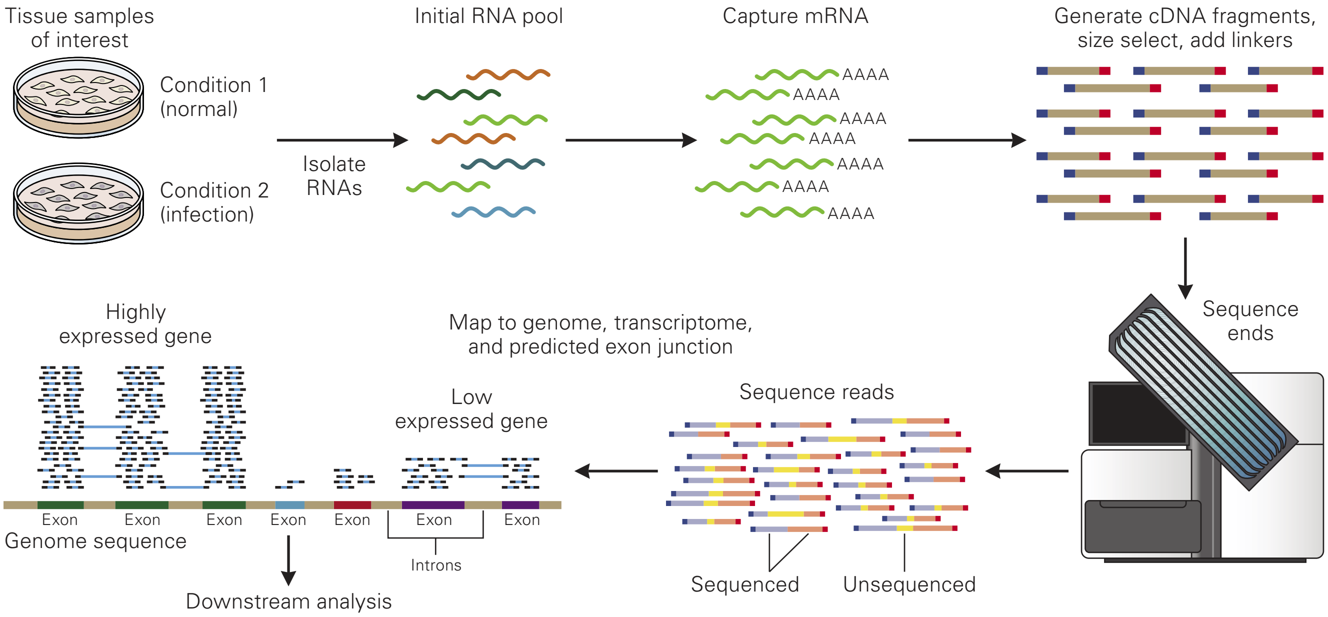


Figure 10-12. Transcriptomic profiling using RNA-Seq technology. The Illumina RNA-Seq platform enables simultaneous and quantitative global expression profiling of all genes in cells or in tissues. For RNA-Seq, the poly-A tail of eukaryotic mRNA is used to capture full-length mRNA transcripts from total RNA extracted from the cells grown under different conditions, and cDNA is made from the mRNA transcripts using reverse transcriptase. The library is directly sequenced by the RNA-Seq technology to yield sequence reads that are aligned with the corresponding genes on the DNA genome template to generate a transcriptional tiling map, enabling determination of mRNA expression levels. Adapted from Griffith M, Walker JR, Spies NC, Ainscough BJ, Griffith OL. 2015. *PLoS Comput Biol* **11**(8):e1004393, with permission.

As an example of the application of RNA-Seq technology toward infection transcriptomics from the host-microbe interface perspective, one study examined the dual transcriptional changes occurring simultaneously in the host and the microbe during the course of infection of human lung alveolar epithelial cells with *Streptococcus pneumoniae,* which is an extracellular pathogen (Figure 10-13). Transcriptional responses of the host cells along with the bacterial responses were monitored over the course of four hours of infection with wild-type encapsulated bacteria and mutant bacteria lacking capsule (deleted in a capsule biosynthetic gene, *Δcps2E*). In each case, total RNA was isolated from both lung cells and bacterial cells and then depleted of rRNAs using rRNA-capture probes attached to beads. The remaining RNA was converted into cDNA and sequenced using an Illumina sequencing platform to yield libraries with about 70 million single-end reads for each of the time points. Sequence reads of 75-nucleotide length were then aligned with a chimeric genome sequence generated by concatenating the *S. pneumoniae* genome as an extra chromosome of the human genome. Subsequent comparative analysis using bioinformatics revealed that host epithelial glutathione-associated genes involved in glutathione biosynthesis, glutathione recycling, and detoxification of reactive oxygen species were activated in response to upregulation of pneumococcal genes responsible for producing reactive oxygen species in both wild-type and mutant bacteria. In contrast to encapsulated wild-type bacteria, the more adherent Δ*cps2E* mutant bacteria repressed (by 3-fold) host epithelial genes associated with innate immune responses, such as *CXCL8* (encoding IL-8, a potent chemoattractant for neutrophils) and *DEFB1* (encoding the

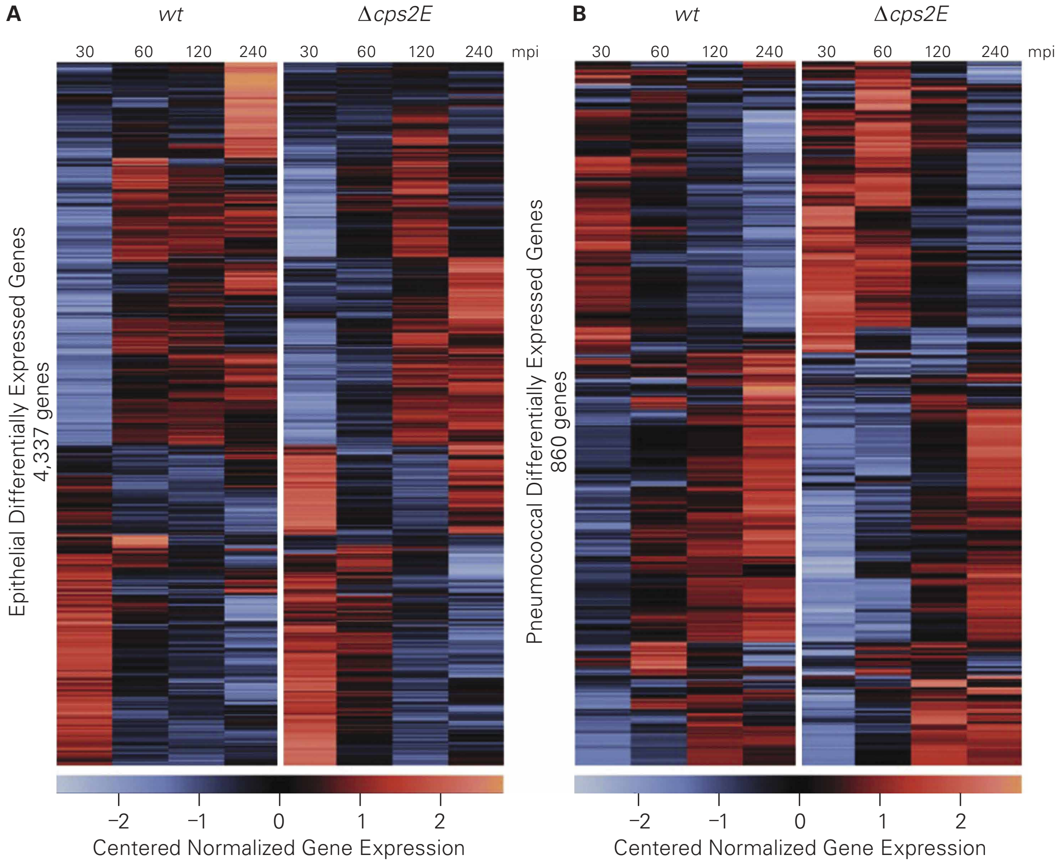


Figure 10-13. Dual RNA-Seq technology enables simultaneous interrogation of both host and pathogen transcriptomes during the course of infection. Human lung epithelial cells were exposed to wild-type encapsulated *Streptococcus pneumoniae* or a mutant strain deleted in the capsule biosynthetic gene, *Δcps2E.* Total RNA from both cells was simultaneously isolated from both cells at 30, 60, 120, or 240 minutes post infection (mpi), depleted of human and bacterial rRNA, converted into cDNA, and then subjected to Illumina sequencing. After processing, each library consisted of about 70 million single-end reads, which were then aligned with a chimeric genome of the human and bacterial genomes. **(A)** Shown is a heat map of the expressed human lung epithelial cell genes in response to the wild-type, encapsulated bacteria (left panel) versus mutant bacteria lacking capsule (right panel) over the time course of infection. **(B)** Shown is a heat map of the expressed bacterial genes in response to exposure to the human cells for the wild-type bacteria (left panel) versus mutant bacteria (right panel). Red denotes upregulated genes, and blue denotes downregulated genes (scale at bottom of heat maps). Reprinted from Aprianto R, Slager J, Holsappel S, Veening JW. 2016. *Genome Biol* **17**(1):198, with permission.

antimicrobial peptide β-defensin-1). These results suggest that loss of the capsule exposes pneumococcal surface factors that bind to the epithelial cells and dampen the innate immune responses, which is an intriguing finding that may have implications for infections where these immune modulating bacterial factors might be released.

Laser-Capture Microdissection (LCM). One problem often encountered when using *in vivo* models of infection is the inability to measure the host responses at the site of infection because the response of the cells directly in contact with the microorganism may be drowned out by responses of other surrounding cells. This interference can sometimes be circumvented by the powerful method of **laser-capture microdissection (LCM)**. LCM is a technique that allows isolation of selected single cells or small groups of cells from tissue sections by means of microdissection (Figure 10-14). A tissue section is placed in contact with a thermoplastic polymer coating (e.g., ethylene vinyl acetate) attached to a transparent film. Using a microscope, the desired cells or cell clusters are identified and the targeted area is exposed to a low-energy, near-infrared laser pulse, which attaches the targeted cells to the polymer coating. The captured cells can then be extracted and their content (RNA, DNA, or proteins) analyzed using various technologies; for example, mRNA expression levels of particular genes of interest can be first converted into cDNA and then qPCR analysis can be performed.

Combining LCM with RNA-Seq technology now also allows researchers to interrogate host response to microbes on a global genome-wide scale. In one such example, gene expression in host neutrophils (PMNs) was characterized in distinct locations (microenvironments) within pneumonic plague lung lesions during infection with *Y. pestis* (Figure 10-15). In this study, the transcriptional responses of neutrophils at the periphery of the lesions, where the neutrophils have just newly arrived at the infection site, were compared to those at the center of the lesions, where the density of bacteria and neutrophils are highest and the cells have been in longer contact with each other. To discriminate transcriptional responses that were specific to those of neutrophils in lungs and not other tissues, transcriptional responses to infection were also compared in neutrophils isolated from bone marrow (BM-PMNs). Results showed that 224 genes were differentially expressed under the four conditions examined (infected BM-PMNs, uninfected BM-PMNs, periphery of lesion, and center of lesion). Further clustering analysis showed that the expression profiles of neutrophils at the center of the highly inflammatory microenvironment of pneumonic lesions resembled that of unstimulated (uninfected) BM-PMNs, suggesting an overall downregulation of the host response (Figure 10-15F). Genes involved in leukocyte migration and apoptotic pathways were downregulated in the lesion center, suggesting that contact with *Y. pestis* favors neutrophil survival. Further analysis showed that this increased survival phenotype was dependent on the T3SS secreted effector protein YopM. This conclusion was confirmed by an experiment showing increased apoptosis in lung lesions of mice infected with the Δ*yopM* mutant of *Y. pestis* compared to that in mice infected with wild-type *Y. pestis*.

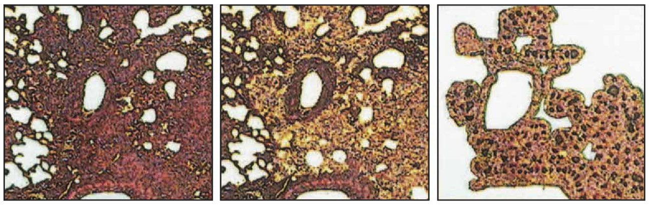
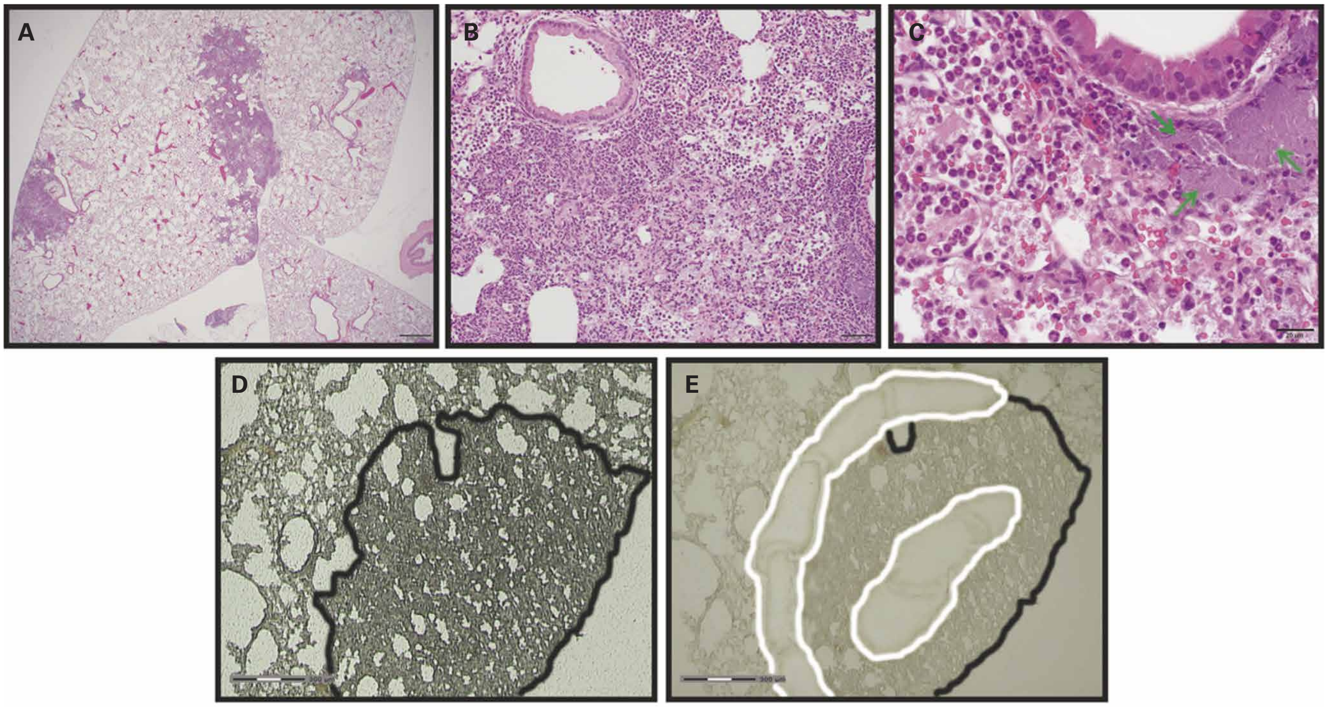


Figure 10-14. Laser-capture microdissection (LCM) plus qPCR analysis to interrogate host responses to infection. Laser-capture microdissection (LCM) and qPCR were used to examine granulomatous tissues from mice infected with *Mycobacterium tuberculosis*. Shown are stained sections of paraffin-embedded lung tissue cells from mice at 42 days post infection with *M. tuberculosis* subjected to LCM to retrieve the characteristic granulomas that form during infection. **Left panel**: before LCM. **Middle panel**: after LCM. **Right panel**: captured granuloma tissue cells. Results from subsequent qPCR analysis of the dissected tissue cells in the right panel provided evidence that conventional use of whole infected lungs to study granuloma-specific gene expression can yield data that may not reflect the actual physiological events that are occurring at the site of the granuloma lesions. Reprinted from Zhu G, Xiao H, Mohan VP, Tanaka K, Tyagi S, Tsen F, Salgame P, Chan J. 2003. *Cell Microbiol* **5**(7):445-453, with permission.

Proteomics

Proteomic profiling has emerged as an important technology complementary to transcriptomics studies, particularly in cases where the host response occurs at the translational or post-translational level. In classic gel-based proteomic analysis, the proteins in a specimen are separated by **two-dimensional (2D) gel electrophoresis** to form a pattern of protein "spots" on the resulting stained gel. Ideally, each spot represents a single protein, which is visualized through colorimetric staining or immunoblotting or is subjected to mass spectrometry analysis for protein identification. Typically, the proteins are first separated by an **isoelectric focusing (IF)** gel on the basis of their **isoelectric point (pI)**. This pI gel is rotated 90° and placed along the top of another gel, where the protein bands in the pI gel are further separated in the perpendicular direction based on their molecular size via polyacrylamide gel electrophoresis (PAGE). Size separation of the proteins can be done under denaturing conditions, where the detergent sodium dodecyl sulfate (SDS) is present in the running buffer (**SDS-PAGE**), or under non-denaturing (native) conditions, where there is no SDS present (**native PAGE**).

Besides being used to determine patterns of protein expression in the bacteria or host, proteomic profiling can also



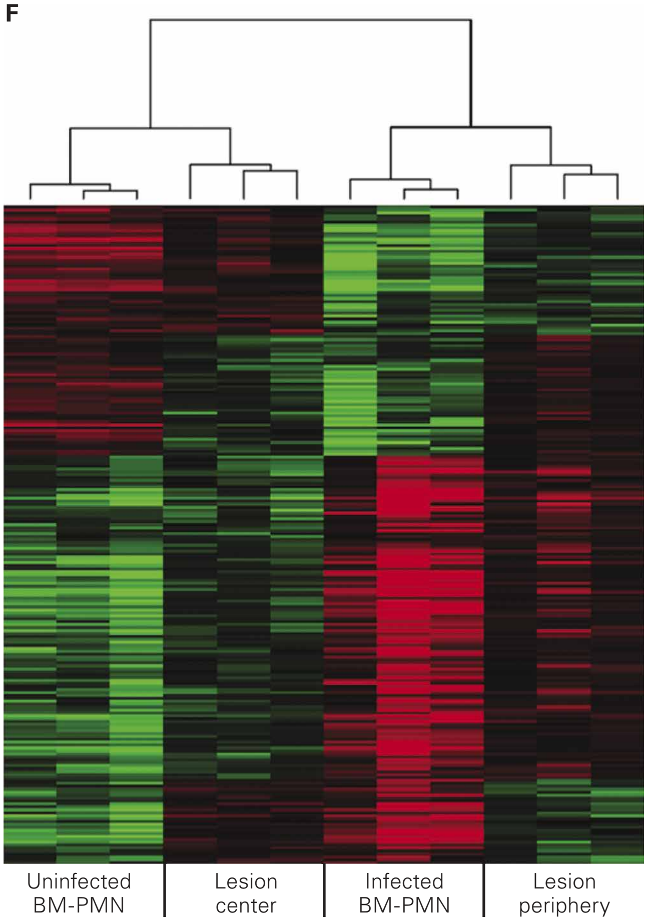


Figure 10-15. Laser-capture microdissection (LCM) plus RNA-Seq analysis to interrogate genome-wide host responses to infection. LCM plus RNA-Seq transcriptomic analysis of neutrophil responses in mouse lung lesions to infection with *Yersinia pestis*. Infection of mice with *Y. pestis* causes formation of distinct inflammatory lesions in the lung consisting of bacteria and mostly neutrophils (PMNs). Panels **A, B,** and **C** show hematoxylin plus eosin (H&E)-stained sections of infected lung at 48 hours post infection at different magnifications (scale bars = 500, 50, and 20 μm, respectively). Hematoxylin stains nuclei blue-violet, while eosin counterstains the cytoplasm and basic extracellular proteins with red. The green arrows in panel C denote dense bacterial aggregates. Panels **D** and **E** show grayscale images (scale bar = 300 μm) of a lung section before (**D**) and after **(E)** the LCM procedure. The black outlines indicate the periphery of the inflammatory lesion where neutrophils are first encountering the bacteria, as opposed to the center of the lesion where the neutrophils have already been in contact with the bacteria for a while. LCM selectively removed cells from within the white-outlined regions of the periphery and center of the lesion. **(F)** Total RNA was isolated from these captured regions and RNA-Seq analysis was performed. In addition, neutrophils residing in bone marrow (BM-PMNs) from uninfected or infected mice at 48 hpi were also isolated and subjected to RNA-Seq for comparison. Shown is a heat map comparing 224 genes (rows) that were differentially expressed, where red denotes increased expression and green denotes decreased expression relative to the mean value of each of the comparisons (performed in triplicate): BM-PMNs from infected versus uninfected mice and from lesion periphery versus lesion center. On top is a dendrogram showing the clustering relationships among the profiles from each sample. Reprinted from Stasulli NM, Eichelberger KR, Price PA, Pechous RD, Montgomery SA, Parker JS, Goldman WE. 2015. *MBio* **6**(5):e01530-15, with permission.

be used as a diagnostic tool. For example, changes in the normal protein pattern in amniotic fluid during pregnancy can indicate the presence of possible infection due to the presence of bacteria; thus, proteomic analysis can be used as a predictor of increased risk of preterm delivery, early-onset neonatal sepsis, or other pregnancy complications due to infection. Proteomic profiling can also be used to identify factors that are recognized by antibodies generated by different hosts during infection (Figure 10-15), and thus can be used to distinguish between different host responses to the same or similar pathogens.

The degree of inflammation can be determined by measuring the levels of certain indicator proteins (called **biomarkers**), such as pro-inflammatory cytokines, present in the fluid. Indeed, multiplex cytokine profiling has become a common diagnostic tool nowadays for assessing the immune status of a patient or experimental subject; discerning the molecular basis of the host inflammatory response and progression of the infection; and characterizing the immunomodulatory effects of bacterial virulence factors and their interactions with host cells. The information gained from the use of cytokine and biomarker profiles has dramatically advanced the development of vaccines, antibiotics, therapeutic drugs, and other treatment modalities.

Gel electrophoresis-based proteomic profiling has had to overcome several disadvantages. Host cell signaling patterns are often complex, and there may be numerous splice variants of some proteins. Therefore, the spot patterns, even for bacterial cells and especially for host cells, which produce many more proteins, are often complicated. Some researchers have overcome this complexity problem by separating and isolating subcellular compartments, such as phagosomes, lysosomes, mitochondria, peroxisomes, or nuclei. Usually, only a portion of the total proteins can be visualized within a range of pIs or molecular sizes, and minor proteins may not be detected.

There have been numerous technological advances that have improved the ease of use, reliability, and reproducibility of gel-based proteomics technology. For example, proteomic analysis can be performed as a comparison of protein amounts from a host cell or tissue isolated from a wild-type or transgenic animal that has been treated with wild-type or mutant bacteria. Previously, this comparison was made by trying to compare and quantify the protein spots from two separate 2D gels. Alignment of the separate gels was difficult and quantification was linear over a very limited range. Current differential 2D-gel approaches have overcome these serious problems through standardization, often using **protein microarrays** (matrices or chips with arrays of specific antibodies, proteins, aptamers, or ligands attached to them) and automation.

As with microarray analysis of transcript amounts (see previous section), comparing 2D gels or probing protein-capture microarrays composed of attached antibodies to

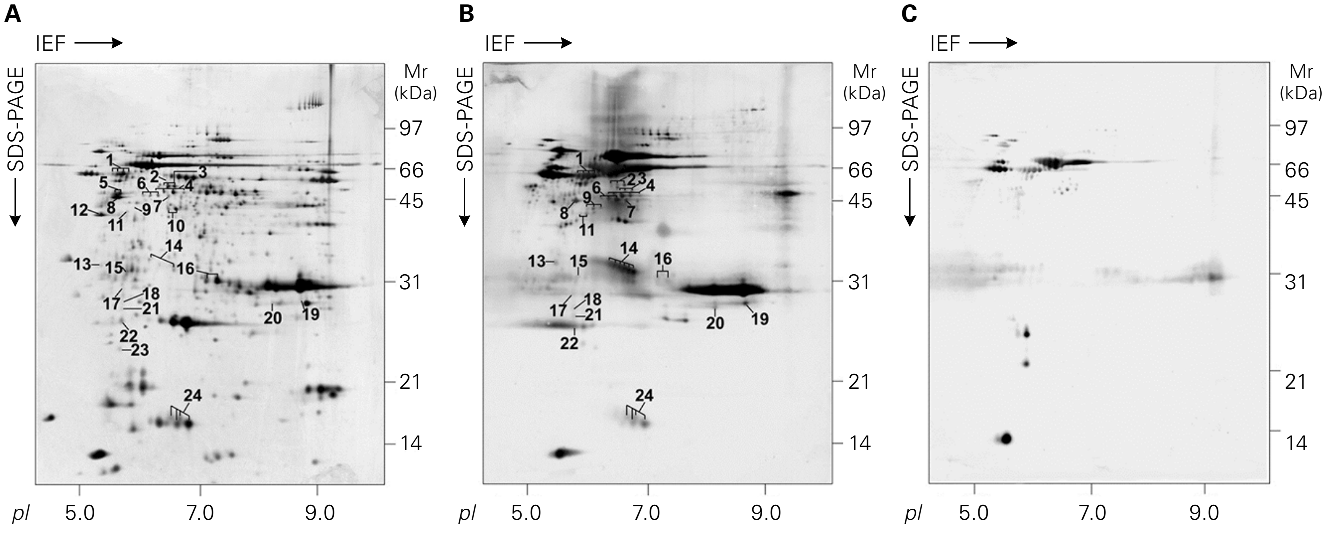


Figure 10-16. Proteomic profiling to identify protein antigens of *Helicobacter pylori* showing greater recognition by anti-sera from gastric cancer patients than from duodenal ulcer patients. Extracts of cell surface proteins from *H. pylori* were separated by 2D electrophoresis using isoelectrofocusing (IEF) with a linear pH 3-10 gradient in the first dimension to separate proteins based on charge, followed by 12.5% SDS-PAGE in the second dimension to separate proteins based on size. The separated proteins were detected by silver staining to see all proteins **(A)** or were transferred to an immunoblot membrane and probed with serum from a patient with gastric cancer **(B)** or duodenal ulcer **(C)** to identify major protein antigens present in patients with each condition. Comparison of immunoblots from 15 patients with gastric cancer with those from 15 patients with duodenal ulcer revealed a number of protein antigens that were recognized by the sera from gastric cancer patients but not by the sera from duodenal ulcer patients. Reprinted from Lin YF, Wu MS, Chang CC, Lin SW, Lin JT, Sun YJ, Chen DS, Chow LP. 2006. *Mol Cell Proteomics* **5**:1484-1496, with permission.

specific proteins involves preparing sample protein extracts from two conditions, labeling each protein extract separately with different colored fluorescent dyes, mixing the labeled extracts together, and either resolving the mixture on a 2D gel or incubating the mixture with the protein-capture microarray. Quantifying changes in relative amounts of proteins bound to the spots becomes much easier by these approaches. The intensity of fluorescence of each spot for the two dyes can be reliably determined, and those spots whose relative amounts increased or decreased can be rapidly identified compared to most spots whose relative amounts did not change.

In addition, robotics coupled with high-end analytical instrumentation has now made the detection and identification of proteins in spots on 2D gels or protein microarrays that detect interacting proteins from extracts routine, rapid, and cost effective. The spots are cut out of the gel, digested with trypsin into small peptides, and subjected to highly sensitive mass spectrometry for analysis. The translated genome sequence is then used to predict the tryptic peptide profiles of all the proteins encoded by the organism (bacterium or host) under study. Computer programs rapidly use these profiles to identify the protein in the spot from the 2D gel.

The advantage of protein microarrays is that the proteins on a protein microarray chip can be directly sampled for binding to proteins in extracts, which can then be identified by mass spectrometry. However, protein microarrays suffer from a major disadvantage, namely the requirement of producing hundreds or thousands of antibodies to specific proteins and the proteins themselves with good yield, purity, and stability (for storage). Nevertheless, many premade protein microarrays are currently available from commercial sources and are rapidly becoming a commonly used platform for interrogating protein-protein interactions; identifying antibody-antigen pairs; identifying host target substrates for bacterial toxins and effector proteins; and profiling hosts immunologic or signaling responses during infection.

Tremendous advances have also been made in gel-less or chip-less proteomics. Again, these cutting-edge, highly sensitive proteomics approaches are based on high-resolution mass-spectroscopy of highly complex protein or tryptic peptide mixtures. However, mass spectrometry without internal mass tags is not quantitative. Therefore, to determine relative amounts of proteins in two extracts, so-called isobaric tags for relative and absolute quantitation (iTRAQ or TMT) must be reacted with tryptic peptide digests, which are then separated by **high-performance liquid chromatography** (**HPLC**). The resulting peaks are analyzed by tandem mass spectrometry (MS/MS) to determine the relative amounts of proteins from the two extracts in a single experiment. LCM technology (described previously) can also be used to selectively capture different cell types from tissue samples, which can then be further subjected to separation of content by HPLC and protein identification by mass spectrometry.

Proteomics technology is being applied as a tool for assessing the host's immune response (**immunoproteomics**) to identify bacterial origins of disease. In chapter 6, it was mentioned that infection with *Helicobacter pylori* is an important risk factor for developing gastritis, duodenal ulcers, and gastric cancer, yet duodenal ulcers and gastric cancer are considered to be clinically quite distinct diseases. So, how then can one go about reconciling these distinctions and proving Koch's postulates for *H. pylori* as the origin of these clinically different diseases? In a variation of *in vivo* induced antigen technology (IVIAT) (described in chapter 9), one approach is to use comparative immunoproteomics to identify bacterial antigens that give common versus distinct host responses associated with the three gastric diseases (gastritis, ulcers, and cancer).

Using this immunoproteomics approach, proteins from *H. pylori* cell extracts were separated on 2D gels (Figure 10-16A), and the separated protein spots were transferred onto membranes. Serum samples from patients with gastric cancer or from patients with gastric ulcers were then used to probe the membranes, and the resulting immunoblots were compared (Figure 10-16B versus 10-16C, respectively). While many of the protein spots were recognized by both serum samples, a number of the spots observed were more strongly recognized or were only recognized by the serum from gastric cancer patients. The researchers found one particular spot that was especially pronounced in gastric cancer patients. This spot corresponds to a host factor that might specifically contribute to gastric carcinogenesis in a subpopulation of presumably predisposed individuals exposed to *H. pylori*.

Metabolomics

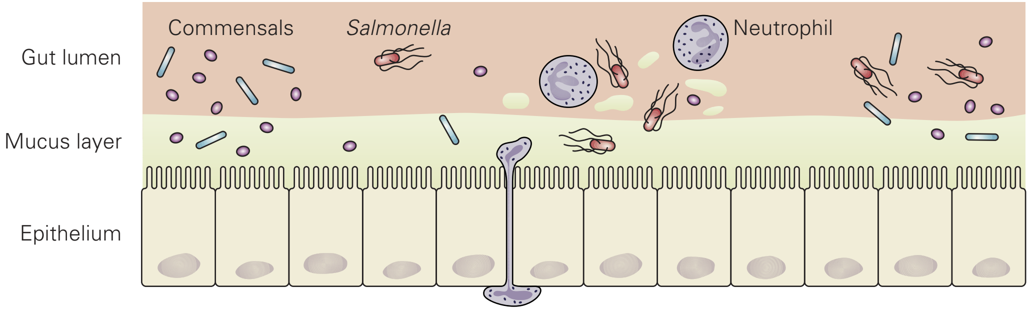
One approach to identification of biomarkers that discriminate healthy from diseased individuals is to gain insights into the functional changes that occur by comparing metabolic profiles (**metabolomics**). Metabolic profiling can be performed at various levels from identifying and quantifying a selected number of metabolites associated with a particular metabolic pathway of interest to genome-wide metabolic reconstructions of entire metabolic networks. The idea is that by quantitatively monitoring perturbations or fluctuations in the metabolite composition of microbiomes (the **metabolomes**) and the host environment (blood, tissues, cells, or fluids) particular signatures or even certain biomarkers will emerge that are indicative or predictive of infection and/or disease outcome. More and more researchers are realizing that there are distinctive associations between certain microbes in a community within the host and the metabolites that are produced by the microbe and the host. These metabolites and their relative composition reflect the state of human cell function, such as the presence of apoptosis, necrosis, or other cytotoxic effects caused by bacterial factors. They can also be indicative of bacterial-induced inflammation or immunosuppression, which can serve to assess the degree of disease susceptibility of the host.

By combining metabolomics with transcriptomics and proteomics, links between genotype and phenotype can be made. For instance, metabolomics can be used to identify the metabolites present, which can then be correlated with the predicted presence of certain enzymes involved in their production through transcriptomics and proteomics. This information can be used to ascertain whether this metabolic pathway is associated with immunomodulation by comparing the levels of proteins and their associated metabolites during the course of infection. This **multi-omics** approach can even be taken a step further to reveal connectivity of multiple metabolic or signaling pathways involved in host response to immune challenges by various pathogens, thereby identifying shared responses for different types of pathogens. For example, a multi-omics approach was used to model host-pathogen-commensal bacterial interactions of the gut microbiome in mice during *S.* Typhimurium infection (Figure 10-17).

Implementing genome-scale metabolic profiling and computationally integrating it with metagenomic, transcriptomic, proteomic, and other profiling approaches to yield comprehensive, predictive models of host-pathogen interactions during infection and under various treatment modalities is still at its infancy—but clearly poised to rapidly advance. While further details of this exciting, developing area of research are beyond the scope of this textbook, we direct the interested reader to some of the selected articles at the end of this chapter for more information.

The Promise and the Caution

Despite the appeal of all of these new genomic tools and high-tech approaches toward identification of virulence factors from both the bacterium and the host, it is important to realize that there are significant limitations and caveats that must be considered. A major problem with any profiling analysis is the large number of "genes of unknown function"



	Pre-infection	Early	Mid	Late
S. Typhimurium	Absent	Primary proliferation	Secondary proliferation; fucose use	Clearance
Host	Homeostasis	Inflammatory response	Glycan release, inflammation	Clearing pathogen
Commensals	Abundant	Decreasing	Low	Increasing
Metabolites	Homeostasis	Sugar accumulation	Sugar accumulation	Regaining homeostasis

Figure 10-17. Model of host-pathogen-commensal microbe interactions in the gut microbiome of mice during the course of infection with *Salmonella enterica* serovar Typhimurium. A multi-omics approach, involving integration of metagenomics, transcriptomics, proteomics, metabolomics, and glycomics (sugar content profiling), was used to develop a model of the interactions among the mouse, the pathogen *S.* Typhimurium, and the commensal microbiota during infection from initial interaction, development of gastroenteritis, and eventual disease resolution. Prior to infection, the gut commensals are abundant and host metabolism is maintaining homeostasis. Introduction and growth of *S.* Typhimurium stimulates an inflammatory response that causes neutrophil activation and disruption of the microbial community structure. This shift in microbial content changes the metabolic content, such that the commensal bacteria no longer metabolize compounds like fucosylated glycans. *S.* Typhimurium responds to the presence of accumulating fucose by increasing its expression of bacterial fucose utilizing proteins. Eventually, the pathogen is cleared from the gut and the normal microbiome environment returns to homeostasis. Adapted from Ansong C, Deatherage BL, Hyduke D, Schmidt B, McDermott JE, Jones MB, Chauhan S, Charusanti P, Kim YM, Nakayasu ES, Li J, Kidwai A, Niemann G, Brown RN, Metz TO, McAteer K, Heffron F, Peterson SN, Motin V, Palsson BO, Smith RD, Adkins JN. 2013. *Curr Top Microbiol Immunol* **363**:21-41, with permission.

that still exist in the genome databases, as well as those genes that are incorrectly annotated. Nonetheless, despite these issues, global genome-wide profiling offers an amazing avenue into further understanding host-microbe interactions, which researchers have just begun to explore. Coupled with the new information gained from multiple analytical approaches (multi-omics), the ability to gain insights about gene functions and even annotate with confidence the remaining genes will become easier, particularly as new technologies, bioinformatics, and data mining tools develop to meet the growing demands.

While systems genetics and multi-omics approaches will undoubtedly play an even greater role in the future, it is also vitally important to bear in mind that a clear hypothesis and critical biologically oriented thinking are still necessary elements to translate the massive amounts of data obtained through these new approaches into coherent models of disease and virulence mechanisms. Ultimately, understanding the complex interactions between pathogens and their host will require a comprehensive knowledge of the role of bacterial virulence factors in disease progression; the host factors subverted by the pathogen to establish infection; the host factors that provide resistance to disease; the prior exposure history of the host with similar or other bacteria and/or other microbes; and the ensuing interplay that occurs between them during infection and subsequent resolution of infection. We are clearly at a critical juncture, where the excitement of discovery, tempered with a little bit of caution, could lead to transformative advancements in our understanding of host-microbe interactions.

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Questions

1. What sort of host factors might be expected to influence the evolution of a pathogen? Explain briefly how each of the techniques described in this chapter could be used to probe the role of a newly discovered host factor (factor X) in the infection process.

2. Following up on your answer to question 1, explain how results of each technique covered in this chapter could DISPROVE the involvement of host factor X in the response to infection by a particular bacterial pathogen.

3. Are any of the experimental approaches described in this chapter useful ONLY for bacterial infections? In that case, where might the approach have to be modified if you were interested in studying the host response to a viral or fungal infection? Which of the approaches could be used to identify host factors that interact with bacterial virulence factors that are secreted?

4. What obstacles do researchers face in the search for host factors that contribute to virulence?

5. Critique this statement: The virulence factors of a pathogen are established by determining how strong or weak a patient's innate and acquired defenses are.

6. What host factors might be involved in determining whether a microbe becomes a pathogen in one individual and a commensal in another? How might these host factors be identified through some of the screening assays talked about in this chapter?

Solving Problems in Bacterial Pathogenesis

1. Research animals serve as essential components with which advances in biomedical research are made. It is now clear that the validity and value of research findings derived from the use of animal models is directly dependent upon their uniform care and physiological health. Thus, it is important for researchers to work with animal care professionals to know and understand what potential infections and/or diseases their animals currently have or have been exposed to in the past. A researcher at your university is interested in developing a new animal model for rheumatoid arthritis that would confirm research findings they have already made in rodents. This researcher has just ordered two different colonies of animals (ferrets and rabbits) to test for suitability

in their studies. Upon arrival at the animal care facility, however, the university veterinarian discovers that most of the animals in each of the animal colonies have contracted bacterial infections, and the veterinarian and researcher have called you, as a microbiologist with expertise in bacterial pathogenesis, to consult and assist with evaluating the impact of these infections on the use of these animals for their research.

In the case of the ferrets, symptoms of disease appear to be anorexia, weight loss, and a massive inflammation of the lining of the gut, with over proliferation of epithelial cells and swelling of local mesenchymal lymph nodes. While several of the animals died due to profound weight loss and blockage of the intestine, most of the animals recovered after 1-2 months. You have identified a new Gram-negative bacterium that was detected by PCR in biopsies of the intestine and lymph nodes of the infected animals. Based on 16S rRNA sequence comparison, you have determined that this new bacterium is related to the bacterium *Lawsonia intracellularis*, which is thus far noncultivatable, and you have named it *Lawsonia ferretii*. So far, you cannot grow *L. ferretii* in laboratory medium, but you can grow it in mouse tissue culture cells, where it appears to be an obligate intracellular pathogen.

a. What immune response was primarily responsible for controlling *L. ferretii* infection in the animals that survived? Be specific.
b. How are *L. ferretii* antigens presented to immune cells? Provide a detailed schematic diagram of the pathway with clear labeling.
c. To further understand the pathogenesis of the intestinal disease in ferrets, you set up an experiment to examine the host response during infection after feeding *L. ferretii* to unexposed ferrets. The results are summarized in the graphs shown in Figure 1. Interpret the results in terms of the host response to infection (i.e., provide a detailed model that explains each of the results shown). Be sure to include your response to part a. in your model.
d. You find that reinfection of animals that have had prior exposure to *L. ferretii* and have cleared the infection results in massive inflammation of the gut, followed by blockage of the intestine and rapid progression to death. Provide a plausible explanation with rationale for this result.
e. When you take serum from animals that survived infection with *L. ferretii* and use the antibodies in western blots, you find that there are several cross-reactive host proteins in uninfected animals. What possible implications do these results have in terms of using these animals for arthritis research? Be sure to provide your rationale.
f. Based on the results from all of the above experiments, do you recommend that the arthritis researchers use the ferrets for their animal studies? Do you recommend that they use this particular colony? Provide your rationale.

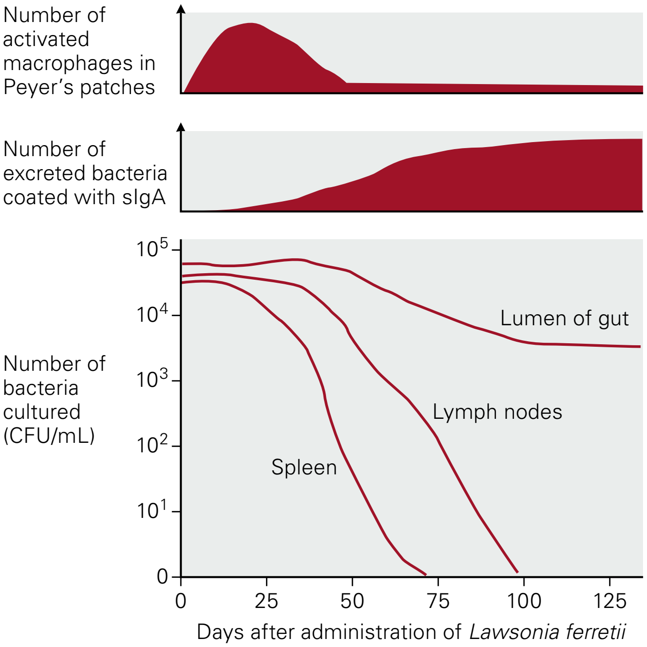

Figure 1. Results.

In the case of the rabbits, you have isolated a new, highly virulent Gram-positive bacterium related to *L. monocytogenes*, which you have named *Listeria leporine*. Pathologic findings are most prominent in the liver and spleen, where you see swelling and necrosis. Clinical signs are generally absent in adult healthy animals, but you find that young and old animals often succumb to systemic infection, including brain lesions and death. To better understand the pathogenesis of the disease, you have developed a mutant screening approach based on existing reagents that have already been developed for *L. monocytogenes*, which includes a temperature-sensitive plasmid (i.e., it integrates into the chromosome when the temperature is shifted to 42°C) containing an erythromycin gene and a promoterless listeriolysin gene, *hly*. From your screening, you identified six genes encoding putative virulence factors, which you have named *llp1* through *llp6*. You then created mutant strains with in-frame deletions in each of these six genes. When administered to the rabbits through their feed, the wild-type bacterium has ID_{50} and LD_{50} values of 10, while all of the mutant strains Δ*llp1* through Δ*llp6* have ID_{50} and LD_{50} values of $>10^9$.

g. Describe *in detail* how you might have identified those six genes using one of the approaches described in chapter 9. Be sure to include a description of all the reagents, conditions, and experimental procedures used, as well as your rationale for why this is the best approach.

h. To verify that the putative virulence factors identified are indeed involved in pathogenesis, what four criteria must be satisfied? (State them in terms of the rabbit infection model.) Provide three *different* modern molecular experiments that could be performed to satisfy these criteria. Be specific!

i. You decide to determine the competitive index of each of the mutants using a young rabbit model. The results for two of the mutants are summarized in Table 1. Determine the competitive index for Δ*llp*1:wt, Δ*llp*2:wt, and Δ*llp*1: Δ*llp*2. Be sure to show how you derived your answer. Interpret your results.

Table 1.

STRAINS USED	# OF BACTERIA IN INOCULUM (CFU MUTANT:CFU WT)	# OF BACTERIA RECOVERED FROM BRAIN ON DAY 10 (CFU MUTANT:CFU WT)
Δ*llp*1:wt	10^5:10^5	10:10^{12}
Δ*llp*2:wt	10^5:10^5	10^2:10^9
Δ*llp*1:Δ*llp*2	10^5:10^5	10^3:10^6

j. To gain some insight into the role of these virulence factors in pathogenesis, you decide to conduct additional experiments. The results are summarized in Table 2. Based on these results, predict the possible virulence factors deleted in Δ*llp1* through Δ*llp6*. Provide your rationale.

Table 2.

MUTANT	GENTAMICIN ASSAY			PLAQUE ASSAY	BRAIN LESIONS	SWELLING OF SPLEEN
	# Colonies without Gentamicin	# Colonies with Gentamicin	# Colonies with Gentamicin and Galactose	Plaques observed	# Colonies on day 10	Increased thickness of spleen section (cm on day 10)
wild-type	10^9	10^8	10^2	Large	10^4	1.5
Δ*llp1*	10^9	10^8	10^2	Large	10^4	0
Δ*llp2*	10^9	10^2	10^2	Large	10	1.6
Δ*llp3*	10^9	10^8	10^2	Small	0	1.5
Δ*llp4*	10^2	1	0	None	0	1.7
Δ*llp5*	10^9	10^8	10^2	Large	0	1.6
Δ*llp6*	10^9	10^8	10^2	Large	0	0

k. Provide one additional experiment that you could perform to confirm your prediction for each gene.

l. Describe a strategy you could use to identify potential transcriptional regulator(s) of the expression of the gene *llp1*. Be sure to provide the reagents, conditions, and how you will know if the regulator(s) is a transcriptional activator or repressor.

m. Based on the results from all of the above experiments, do you recommend that the arthritis researchers use rabbits for their animal studies? Do you recommend that they use this particular colony? Provide your rationale.

n. Your curiosity has been piqued about the underlying host factors that drive the differences in host responses to infection by the different bacteria observed between the animals. Describe how you would go about setting up an experimental screening system that could help identify some of these host factors.

2. *Burkholderia pseudomallei* is a Gram-negative bacterium that causes the disease melioidosis. Clinical manifestations of melioidosis vary greatly from acute or chronic lung pneumonia to overwhelming lethal septicemia. The most common clinical presentation is chronic pneumonia, which is found in about half of the patients showing symptoms of melioidosis. An important risk factor for melioidosis was identified in a number of near-drowning survivors following the tsunami of December 26, 2004 in southern Thailand. A group of researchers hypothesized that direct aerosol delivery of *B. pseudomallei* into the lungs may result in the enhanced ability of this pathogen to cause disease. The researchers wanted to develop two animal models of disease, one that

mimicked the acute form of disease that usually results in lethal septicemia if untreated and one that mimicked the chronic form of disease. To do this, they tested three different clinical isolates of *B. pseudomallei* (Bp#1, Bp#2, and Bp#3) in a rat lung model of infection, where the bacterium was directly introduced into the left lung using laparoscopy and disease progression from the left lung to the right lung as well as systemic infection in the blood was monitored via isolation of bacteria from the lungs and blood of the survivors at each time point, followed by plating on agar and counting the number of bacterial colonies (CFUs per left or right lung or per mL of blood). The results are shown in Table 3.

Table 3.

ISOLATE DOSE	SURVIVORS AT 2 DAYS (20 PER GROUP)	SURVIVORS AT 28 DAYS (20 PER GROUP)	# CFUs LEFT LUNG AT 2 DAYS	# CFUs LEFT LUNG AT 28 DAYS	# CFUs RIGHT LUNG AT 2 DAYS	# CFUs RIGHT LUNG AT 28 DAYS	# CFUs BLOOD AT 2 DAYS	# CFUs BLOOD AT 28 DAYS
Bp#1								
10^1	20	0	10^8	–	10^8	–	10^8	–
10^2	18	0	10^8	–	10^8	–	10^9	–
10^4	8	0	10^{10}	–	10^8	–	10^{10}	–
10^6	4	0	10^{10}	–	10^{10}	–	10^{10}	–
10^8	2	0	10^{10}	–	10^{10}	–	10^{10}	–
Bp#2								
10^1	20	18	10^2	10^3	0	10^2	0	0
10^2	20	16	10^3	10^4	0	10^2	0	0
10^4	20	4	10^7	10^8	0	10^3	0	0
10^6	20	2	10^8	10^7	10^2	10^6	10	0
10^8	20	1	10^8	10^9	10^2	10^6	10	0
Bp#3								
10^1	20	20	10^2	10^3	0	0	0	0
10^2	20	20	10^3	10^3	0	0	0	0
10^4	20	20	10^8	10^3	0	0	0	0
10^6	20	19	10^{10}	10^3	0	0	0	0
10^8	20	18	10^{10}	10^3	0	0	0	0

a. Determine the LD_{50} and ID_{50} values for each of the isolates (Bp#1, Bp#2, and Bp#3) at 2 days and 28 days post-inoculation (use the left lung data for the ID_{50} values). Be sure to show how you derived your answers. Interpret your results.

b. Considering all of the above information, which of the *B. pseudomallei* isolates would be most suitable for use in an infection model of chronic melioidosis? Which would be best for an infection model of acute melioidosis? Be sure to state your rationale for each.

c. When isolates of each of the bacteria were injected directly into the rats either intraperitoneally or intravenously, Bp#1 and Bp#2 did not result in death of any of the rats even at doses as high as 10^8, and the animals showed no signs of distress or infection after 3 days. However, Bp#3 killed all of the rats by 2 days after injection. Are these results consistent with those found for direct lung inoculation? Provide your rationale and suggest an explanation for this observation. Do these results support the hypothesis put forth by the researchers regarding the role of aerosolization in disease outcome? Be sure to state your rationale.

d. Starting from initial inoculation until 28 days post-inoculation, describe *in detail* the processes that you would expect to be involved in the host immune response to isolate Bp#3 in an unimmunized tsunami near-drowning victim. Be sure to include both the innate and adaptive immune responses. [Hints: Use the results from the animal infection model as a guide. How are the immune pathways activated upon initial exposure? How are the antigens presented? How are the bacteria cleared or controlled? You may use a schematic diagram so long as everything is clearly labeled.] Describe how you might monitor these processes during infection. What factors or responses would you specifically measure?

e. You are interested in identifying host factors that are involved in mediating the acute disease versus those involved in mediating the chronic disease. Describe how you would go about identifying these host factors.

11

IN THIS CHAPTER

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- Biofilms
- Motility and Chemotaxis

Colonization of Host Surfaces

- Penetrating Intact Skin
- Penetrating the Mucin Layer
- Evading the Host's Innate Immunity
- Nutrient and Iron Acquisition Mechanisms
- Adherence

Evading the Host Immune Response

- Avoiding Complement and Phagocytosis
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CHAPTER 11

Bacterial Strategies for Colonization and Survival in the Host

In the case of serious crimes, police sometimes call in profilers, specialists who analyze what is known about the crime, especially the MO (*modus operandi*) of the criminal, and attempt to deduce the characteristics of the perpetrator. Microbiologists who investigate infectious diseases have recourse to similar types of specialists: the basic scientists who attempt to deduce the detailed traits that characterize a particular "microperp" and explain how a disease is caused by the bacterium (MO). The traits that go into such a microbial profile extend beyond superficial traits, such as Gram stain status, to more detailed characteristics called virulence factors. These factors explain why, in many cases, members of the same species do not cause disease—only a subset of the strains possesses the necessary virulence factors. Also, as expected from the fact that different microperps have different MOs, the set of virulence factors employed by one microperp differs from the set of virulence factors employed by another.

What Does Not Kill You Makes You Stronger—Or, a Better Pathogen

Just as the body has evolved multilayered strategies for defending itself against bacterial invasion, bacteria have evolved their own strategies for countering the body's host defenses. The ability of pathogenic bacteria to cause infection and disease is the sum of structural, biochemical, and genetic characteristics that contribute to a microbe's pathogenicity. As noted in chapter 9, **virulence factors** are broadly defined as molecules produced by or strategies used by pathogenic bacteria to cause disease, including those that enable the bacteria to achieve attachment and colonization of the host, evasion or survival of the host's immune system, invasion and dissemination in the host, acquisition of nutrients and growth within the host, and release and spread to other hosts.

In this chapter, we explore some common themes seen in bacterial strategies for penetrating, evading, and surviving the defenses of the human body. We will begin this chapter by covering some general features of bacterial pathogens that enable them to survive in the external environment and then colonize and infect the human body in the presence of a myriad of host defenses (Table 11-1). We have placed one group of important bacterial virulence factors—toxins and other factors produced by the bacteria and released into the medium that bind and enter the host cell, or that are directly delivered into the host cells, that then damage eukaryotic cells and tissues—in a chapter of their own (see chapter 12). Many bacteria have also developed or acquired resistance to antibiotics or disinfectants. These virulence properties are discussed in chapters 15 and 16.

In order to invade and survive in a host and establish an infection, a pathogenic microbe must be able to do the following:

1. Attach to host cells for colonization
2. Evade the host's innate and adaptive immune defenses and persist in the host
3. Obtain iron and other nutrients needed to multiply, especially those that are essential for growth but may be limiting within the host
4. Disseminate or spread within a host and to other hosts (this is critical for the survival of the bacterial species)
5. Produce symptoms of disease in the host in order to be considered pathogenic (although production of symptoms is not necessarily a requirement in and of itself, disease is often a result of the presence of the microbe and/or its products or the host's response to the presence of the microbe and/or its products)

Table 11-1. Mechanisms used by bacteria to facilitate colonization and survival in a host

VIRULENCE FACTOR/STRATEGY	FUNCTION
Biofilm formation	Ability to bind to surfaces and establish multilayered bacterial communities; reduced susceptibility to antibiotics; bacteria continue to dissociate and disseminate through body
Motility and chemotaxis	Reaching mucosal surfaces (especially areas with fast flow or with nutrients)
Siderophores, surface proteins that bind transferrin, lactoferrin, and other iron-binding proteins	Iron acquisition
Iron abstinence	A few bacteria (e.g., *Borrelia burgdorferi*) have replaced iron-requiring enzymes with similar ones that use manganese instead
Transition metal ion uptake and efflux	Homeostasis of essential cations, including zinc (Zn^{2+}) and manganese (Mn^{2+})
Capsules (usually polysaccharide)	Prevent phagocytic uptake; reduce complement activation
Lengthened and shortened LPS O antigen	MAC not formed; serum resistance
sIgA proteases	Prevent trapping of bacteria in mucin
C5a peptidase	Interferes with signaling function of complement component C5a
Pili and fimbriae	Adherence to mucosal surfaces
Nonfimbrial adhesions	Tight binding to host cells
Binding to M cells	M cells used as natural port of entry into body
Variation in surface antigens	Evade antibody response
Invasins	Force nonphagocytic cells to engulf bacteria
Actin rearrangements in host	Induce phagocytosis, movement of bacteria within host cells, cell-to-cell spread
Catalase, superoxide dismutase	Reduce strength of oxidative burst to allow survival in phagosomes
Elastase and other proteases	Degrade extracellular matrix proteins to allow dissemination through body
Nucleases	Degrade DNA released from host cells to reduce viscosity and to allow dissemination through body
Toxic proteins (toxins)	Kill phagocytes; reduce strength of oxidative burst; poke holes in host cells to release cell contents for iron and nutrients; modify host cell signaling to dampen immune response; cause cell morphology changes that increase dissemination; modify host cell processes to allow intracellular survival and replication (see chapter 12)

A useful paradigm for thinking about the three stages of bacterial infection (colonization, persistence, and spread) is a comparison to an *in vitro* growth curve (Figure 11-1). Typically, bacterial growth in culture can be divided into distinct phases: (1) an initial lag phase, (2) a logarithmic or exponential phase, (3) a transition to early exponential phase, and (4) a stationary phase that, after a period of time, may be followed by (5) a death phase. For some bacterial species, genes needed for colonization, such as those encoding adhesins (attachment proteins), are turned on during the lag phase to promote close associations with host cells. Genes required for persistence, such as those encoding proteins required to withstand the immune response, are turned on in the exponential or transition phases. Genes encoding toxins and degradative enzymes required for spread through the body are often turned on in the stationary phase. In addition, some bacteria enter a long-term survival state upon entering the stationary phase (dormancy), while others form endospores that can germinate later, and still others lyse themselves (autolysis), presumably to exchange genetic material and trigger host inflammatory responses. This model provides the perspective that bacterial virulence progresses in distinct stages, similar to those of a standard growth curve.

Many strategies used by bacterial pathogens to establish infection in a host probably began evolving long before animals and humans were even blips on the evolutionary radar screen. The majority of bacterial defense strategies would have been useful for survival in natural environments (e.g., adherence to surfaces to stay close to a promising food source) or as defenses against voracious protozoa (e.g., the ability to avoid or survive phagocytosis by neutrophils or macrophages). Perhaps the reason that intact skin and the antibody response are such effective defenses is they are very recent evolutionary developments that have no counterpart in protozoa. Still, even these defenses can be compromised. Microbes that use insect vectors or take advantage of wounds have ways to bypass the skin barrier, and microbes that can rapidly change their surface proteins or produce antibody-degrading enzymes have developed methods for bypassing the specific defenses of the immune system.

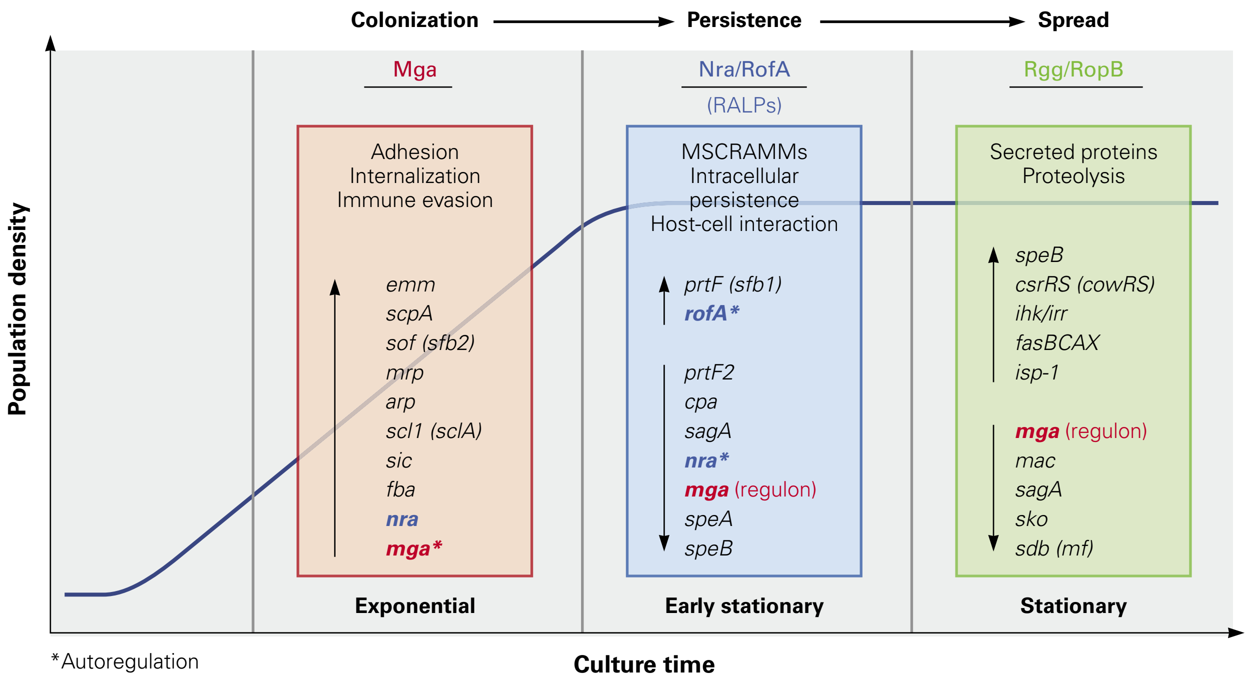

Colonization
Persistence
Spread
Mga
Nra/RofA
(RALPs)
Rgg/RopB
Adhesion
Internalization
Immune evasion
emm
scpA
sof (sfb2)
mrp
arp
scl1 (sclA)
sic
fba
nra
mga*
MSCRAMMs
Intracellular persistence
Host-cell interaction
prtF (sfb1)
rofA*
prtF2
cpa
sagA
nra*
mga (regulon)
speA
speB
Secreted proteins
Proteolysis
speB
csrRS (cowRS)
ihk/irr
fasBCAX
isp-1
mga (regulon)
mac
sagA
sko
sdb (mf)
Population density
Exponential
Early stationary
Stationary
Culture time
*Autoregulation

Figure 11-1. A model showing the correlation between virulence gene expression and the stages of a typical bacterial growth curve. The model emphasizes the ordered progression of bacterial gene expression in *Streptococcus pyogenes* (group A strep [GAS], which causes several serious human diseases, including strep throat) that enables colonization, persistence, and spread. Mga (red), Nra/RofA (blue), and Rgg/RopB (green) are stand-alone transcriptional regulators that mediate gene expression in each stage of growth or infection. Upward and downward arrows indicate increased or decreased expression, respectively, of the indicated genes. Autoregulated regulons are indicated with asterisks. MSCRAMMs stands for microbial surface components recognizing adhesive matrix molecules of the host. From Kreikemeyer B, McIver KS, Podbielski A. 2003. *Trends Microbiol* **11**:224–232, with permission from Elsevier.

Preinfection

Survival in the External Environment

Bacteria are defined by their environment and are constantly adapting and evolving to survive in the environments in which they reside. Until recently, researchers assumed that the most important environment shaping pathogen evolution has been the human or animal host. However, this simplistic view is now coming into question with the accumulation of evidence that strongly suggests the environment outside the human host might play a much greater role than was previously thought for many pathogens. Factors that limit bacterial survival in external environments also restrict their access to the host. The flip side to this scenario is that bacteria that do survive in these harsh external environments might acquire—through mutation and natural selection or horizontal gene transfer—factors that make them better able to survive and establish infection in the host.

Over the past decade, an increasing number of researchers have begun to appreciate the fact that, while some bacterial pathogens move directly from human to human or between humans and animals, a number of them also spend long periods of time in the external environment. They must not only be able to survive in the external environment, but must also be able to survive the abrupt transition to the very different environment of the host body. In natural environments outside the host body, bacteria encounter five major problems: availability of essential nutrients, lack of adherence sites similar to those found in the host, exposure to noxious chemicals, exposure to sunlight and weather extremes, and the need to avoid predatory protozoa and deadly bacteriophage.

To deal with these stressors, some Gram-positive bacteria, such as *Clostridium* and *Bacillus* species, form **endospores** that protect them against unfavorable environmental conditions, including temperature extremes, desiccation, UV light, high or low pH, high or low osmolarity, and many potentially harmful chemicals. Even bacteria that do not produce spores can be resistant to desiccation and other environmental stressors. Examples of these are staphylococci and enterococci, which are inherently able to limit protein oxidation and DNA damage during dehydration and are thus more tolerant of desiccation.

Bacteria that can attach to abiotic surfaces or to each other through formation of colonies or biofilms (discussed in the following section) are better able to survive in their environment and form a significant reservoir. These reservoirs serve as the sources for many pathogens that we encounter. Bacteria can also protect themselves by converting to a metabolically reduced or dormant state. Finally, some bacteria simply parasitize another host, such as an insect or amoeba, that protects and sustains them. How pathogenic bacteria that have a reservoir outside the host make the transition to the conditions inside the body varies depending on the nature of the reservoir (e.g., water or an invertebrate). For example, the intracellular Gram-negative *Legionella pneumophila*, the causative agent of Legionnaire's disease, can replicate within specialized endoplasmic reticulum (ER)-derived vacuoles in lung alveolar macrophages. In the external environment, the bacteria live within free-living protists, such as amoebae, *Tetrahymena,* and *Paramecium*. Evidence suggests that *L. pneumophila* have a mechanism that enables them to switch endosymbiotic lifestyles, adapting to a suitable host environment or escaping the host if the environment is not favorable.

A number of bacteria produce toxic substances known as **secondary metabolites**, which include acids from fermentation processes, antibiotics, bacteriocins, or antimicrobial peptides. To protect against these noxious substances that they themselves or other bacteria have produced, some bacteria have developed adaptive strategies to enhance their metabolic diversity or to alter their membrane properties. Bacterial pathogens typically produce protein factors that modify, degrade, or remove (pump out) the noxious substances. For example, many pathogens have membrane-spanning efflux pumps in their cytoplasmic membranes that transport toxic substances from the bacterial cytoplasm back out into the environment. These efflux pumps are often recruited to remove antibiotics and are associated with antibiotic resistance (see chapter 16).

Genome plasticity, an evolutionary feature of many bacterial genomes that enables DNA exchange and adaptation to changes in environmental conditions, is thought to contribute to the development of increased metabolic diversity and altered membrane properties. Since nutrients are typically limited in external environments, most bacteria that spend at least some of their time outside the host body have the ability to metabolize a variety of substrates and have evolved uptake mechanisms to enhance nutrient acquisition where nutrient concentrations are very low.

Biofilms

Many bacteria are able to form **biofilms**. As the name suggests, biofilms are dense, multiorganismal layers of bacterial communities attached to surfaces. They are refractory to treatment with antibiotics or disinfectants, and their formation protects against phagocytic attack by protozoa in the external environment and by phagocytes of the host immune system. In biofilms, bacterial attachment to a surface and to each other is often mediated by an **extracellular polysaccharide** (**EPS**) slime that acts as a type of "glue" to hold the community together. Sometimes this slime is also composed of secreted proteins or released extracellular DNA. A biofilm is more than just a bunch of glued-together bacteria, however. At one time, scientists thought of biofilms as a relatively simple, uniform, multilayered community of bacteria, but it is now clear that biofilms exhibit a developmental sequence and have a complex architectural structure that looks more like mushroom-shaped islands of bacteria separated by channels that allow nutrients to reach most of the biofilm community (Figure 11-2).

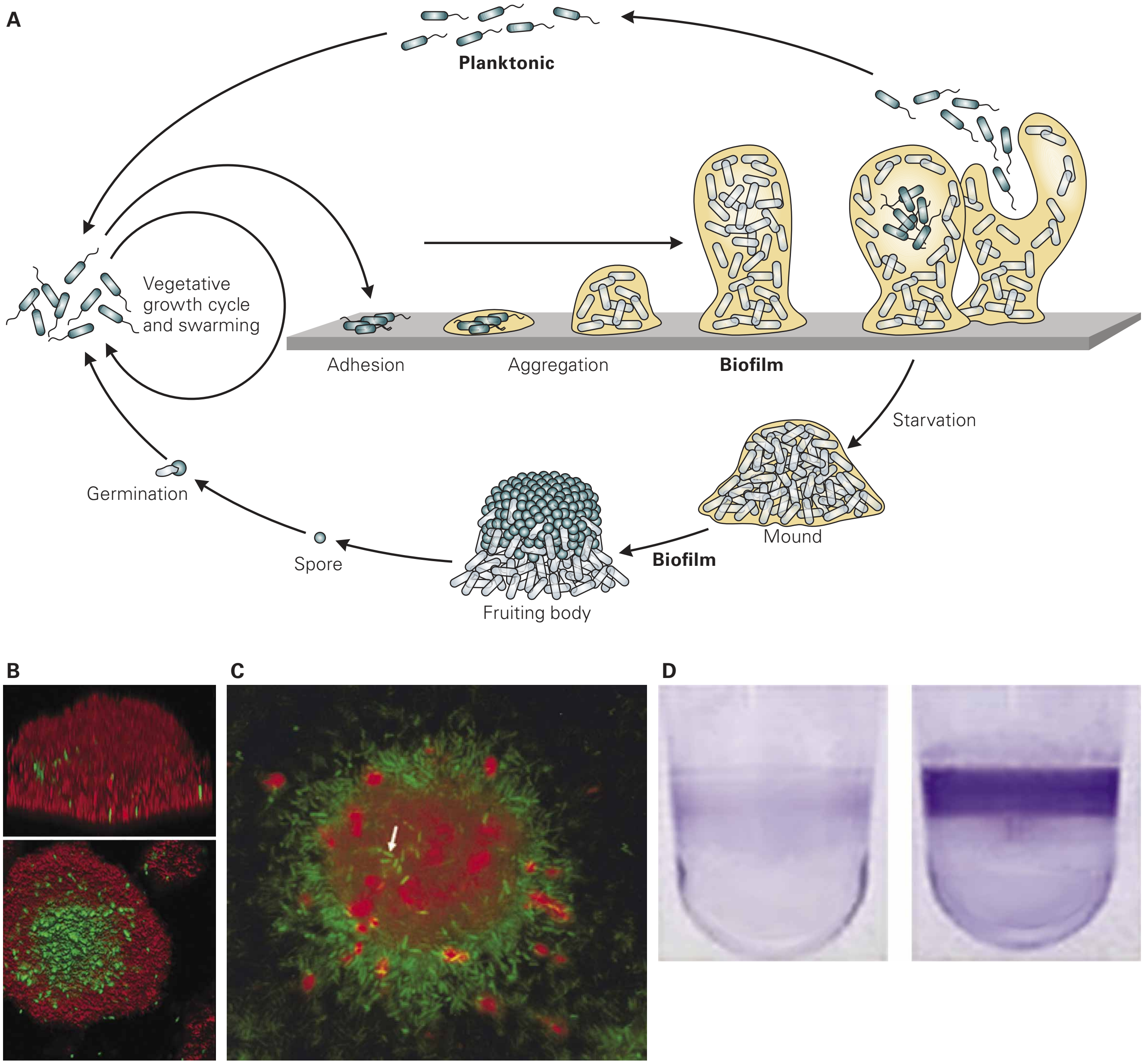


Figure 11-2. Developmental life cycle of biofilms. (A) Biofilms have a developmental sequence with a complex architectural structure that includes attachment to a surface and to each other mediated by an extracellular polysaccharide slime that holds the community together, as well as a free-swimming planktonic stage for some bacteria, such as Gram-negative *Vibrio* and *Pseudomonas* species, and sometimes an endospore/spore stage for surviving harsh environmental conditions, such as Gram-positive *Bacillus* and *Clostridium* species. **(B)** Attachment of bacteria (stained with green dye in the image) to a surface and to each other is mediated by an extracellular polysaccharide (stained red in the image) that holds the community together. Reprinted from Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. 2010. *Mol Microbiol* **75**:827–842, with permission. **(C)** Cell death occurs in localized regions within microcolonies of biofilms. Shown is a confocal micrograph of a *Pseudomonas aeruginosa* biofilm, where live cells are stained green and dead cells (with compromised cell membranes) are stained red. A subpopulation of cells inside the microcolony is also still viable (arrow). Reprinted from Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, Givskov M, Kjelleberg S. 2003. *J Bacteriol* **185:**4585–4592, with permission. **(D)** Biofilm formation can be detected and measured by staining with the dye crystal violet, which binds to polysaccharides of extracellular matrix and capsules. Shown are crystal violet-stained bacterial biofilm residues that form a dark purple ring around the culture tube at the medium-air interface for a bacterium that forms a biofilm (right panel), compared to one that does not form a biofilm (left panel). Reprinted from Heussler GE, Cady KC, Koeppen K, Bhuju S, Stanton BA, O'Toole GA. 2015. *MBio* **6:**e00129-15, with permission.

Biofilms contribute to different types of medical problems. For example, in the case of Legionnaires' disease, where air-conditioning units created aerosols containing *L. pneumophila,* that caused a new type of lung disease, biofilms on the walls of the air-conditioning cooling towers were probably one source of the aerosolized bacteria. The **plaque** that causes dental caries and periodontal disease is actually a biofilm made mainly of aerobic bacteria when it is above the gums and anaerobic or facultative anaerobic bacteria when it is below the gum. Plaque that is not removed becomes mineralized over time to form calculus (tartar). Dislodging plaque is not a trivial task, as anyone who has visited a dentist's office for tooth scraping or gum cleaning knows very well. If not removed regularly, the plaque can lead to dental caries, irritated and inflamed gums (gingivitis), or even periodontal disease. Those bacteria closest to the tooth surface switch to anaerobic respiration, which leads to the release of acids that in turn can lead to demineralization of the tooth surface and dental caries. Irritation of the gums around the teeth where the plaque is located can also lead to gingivitis, periodontal disease, and eventually, tooth loss. Gum disease can even lead to fatal infections, if certain oral bacteria enter the bloodstream and cause endocarditis by attacking the heart (see Box 11-1).

Another type of medical biofilm problem is the formation of biofilms on external and internal body surfaces, particularly mucosal surfaces like those found in the lung or inner ear or on wound areas like severe burns. *Pseudomonas aeruginosa* is notable for its ability to form biofilms, and it has been used as a model organism for studying biofilm formation and structure. Cystic fibrosis patients have recurrent lung infections with *P. aeruginosa* that ultimately result in enough lung damage to cause death. Biofilm formation by *P. aeruginosa* is thought to be a factor in lung infections of cystic fibrosis patients because the bacteria appear to form biofilm-like assemblages in the lung that are difficult to treat with antibiotics. *P. aeruginosa* is also known for its ability to cause death in burn patients, who survive the initial burn trauma but succumb to later infection. In addition to lung infections caused by lung mucosal damage due to inhaling fire-heated air, the badly burned skin surface area can also be colonized by *P. aeruginosa* to form a biofilm and, since the normal defenses of the skin are breached at these sites, the bacteria can leave the biofilm and enter the body.

Biofilms can also cause problems on medical devices, such as catheters, that breach the skin. Biofilms form on plastic tubing (e.g., venous catheters, urinary catheters) and on contaminated plastic implants (e.g., heart valve or hip joint replacements). Because catheters breach important defensive barriers, such as skin or the urethral sphincter, they can transmit bacteria into the body and serve as a constant source of infection. Although plastic is a modern human invention, some bacteria rapidly adapted to this novelty and have become quite adept at forming biofilms on it. Biofilms on plastic implants, whether caused by contamination at the time of insertion or seeded by transient bacteremia (bacteria in the bloodstream), create a serious medical problem. The bacterial communities within these biofilms are refractory to antibiotic treatment, and formation of the biofilm gives the bacteria a measure of protection against phagocytic cells. Bacteria in the interior of the biofilms are also protected from the forces of fluid flow in many body sites and are thus resistant to expulsion from the body and can persist for long periods of time. However, because cells can break off from the biofilm and enter the bloodstream, biofilm-encrusted implants are ticking time bombs that may "explode" into septic shock at any moment.

It is very difficult to eliminate a biofilm once it is established. Plastic implants that acquire a bacterial biofilm usually must be removed and replaced surgically because antibiotics are not effective at sterilizing them. It is still not well understood why bacteria that are susceptible to an antibiotic if they are free-living become more resistant to the same antibiotic when growing in a biofilm. One possible explanation is that bacteria in a biofilm are in a relatively inert metabolic or slow-growing state that makes them less susceptible to antibiotics, most of which work best on rapidly growing cells. Also, those antibiotics that simply stop bacterial growth rather than killing the bacteria outright rely on the accessibility of the stalled bacteria to phagocytic cleanup for effectiveness, but the extended size of the biofilm structure makes engulfment by phagocytes almost impossible.

Motility and Chemotaxis

Movement through fluids, such as water, urine, and intestinal contents, is mediated by bacterial structures called **flagella**, long (up to 20 µm) helical structures that extend outward from the surface of the cell (Figure 11-3A). A flagellum consists of a hollowed tube (**filament**) with a diameter of about 20 nm that is formed by polymerized subunits of a protein called **flagellin**. Each flagellum is attached to the cell surface by a hook-shaped protein complex. A total of about 25 proteins make up the flagellum and its rotary motor complex, which spans the inner membrane, periplasm, and outer membrane (Figure 11-3B). This hook structure connects the flagellum to the cell and the motor structure (**basal body**) is embedded in the cytoplasmic membrane. The motor that provides flagellar rotation is powered through **proton motive force** (i.e., energy generated by the flow of protons across the cytoplasmic membrane due to a proton concentration gradient [proton pump] or, in some cases, a sodium ion gradient [sodium ion pump]). Since the flagellum has a helical structure, rotation makes it act like a corkscrew and propels the bacterium through the liquid environment.

Flagella form left-handed helices; counterclockwise rotation of a single flagellum will push the bacterium forward.

Box 11-1.

Disease without Virulence Factors: Subacute Bacterial Endocarditis

The title of this box seems self-contradictory. After all, virulence factors are associated with microbes that cause disease. Yet this anomaly has arisen in the case of infections caused by opportunistic pathogens. Subacute bacterial endocarditis is a good example of such opportunistic infections and illustrates the difficulty in defining what is a virulence factor for such bacteria.

Endocarditis is an infection of the heart valves, which can be fatal due to destruction of the valves and surrounding heart tissue. One type of bacterial endocarditis, called subacute because it develops more slowly than endocarditis caused by more virulent bacteria, is caused by a group of streptococci that are normally found in the human mouth (α-hemolytic streptococci). The α-hemolytic streptococci are part of the resident microbiota of the mouth and are not normally able to cause disease. However, in people with prior heart valve damage due to rheumatic fever or congenital valve defects, α-hemolytic streptococci can be deadly.

During dental surgery, large numbers of oral bacteria can enter the bloodstream. Phagocytes in the blood rapidly destroy these bacteria, including the α-hemolytic streptococci. In people with heart valve abnormalities, the turbulent flow of blood near the abnormal valve causes loose clots consisting of fibrin and platelets to form on the valve surface. These clots are called "vegetation." Oral streptococci that manage to reach the heart valve and enter these vegetations are protected from phagocytes, which cannot penetrate the protein network of the vegetation. Accordingly, the bacteria can survive and grow in these sites. It is not clear what causes the damage to the heart valve. Complement activation triggered by the bacteria may cause some damage, and phagocytes attracted to the area but unable to get to the bacteria may also cause tissue destruction. Proteases produced by the bacteria themselves may also make a contribution.

Thus, a completely "avirulent" bacterium can cause a fatal infection in the right kind of host, and a person who dies of subacute bacterial endocarditis is just as dead as someone who dies of typhoid fever or other infections caused by the so-called "virulent" bacteria, which have classical virulence factors. The α-hemolytic bacteria in the mouth are highly susceptible to killing by penicillin. So, when the connection between dental surgery, heart valve damage, and subacute bacterial endocarditis was first made in the late 1950s, dentists were advised by the American Heart Association (AHA) to give patients with damaged heart valves penicillin prophylactically before and after dental surgery as a way of preventing the disease. However, over the decades since, cumulative scientific evidence suggested that there was little to no support of the effectiveness of antibiotic prophylaxis in preventing bacterial endocarditis. In 2007, the AHA subsequently altered their guidelines to limit the use of antibiotic prophylaxis to only very high-risk cases, but controversy remains and many physicians still prescribe antibiotics for patients outside the recommended guidelines.

A possible explanation for the apparent ineffectiveness of antibiotics in preventing bacterial endocarditis is the fact that the heart surface is not highly vascularized. Since antibiotics are delivered to a site by blood vessels, it may be difficult to achieve sufficient concentrations of antibiotics in some regions of the heart despite the fact that large quantities of blood flow through the chambers of the heart itself. Also, the bacteria undoubtedly form a biofilm, which has reduced susceptibility to antibiotics. Whatever the reason, α-hemolytic streptococci not only provide an example of disease without special virulence factors, but also an unexpected example of the failure of antibiotic therapy without special antibiotic resistance mechanisms.

Source:

Lockhart PB, Hanson NB, Ristic H, Menezes AR, Baddour L. 2013. Acceptance among and impact on dental practitioners and patients of American Heart Association recommendations for antibiotic prophylaxis. *J Am Dent Assoc* **144**:1030–1035.

Similar rotation of multiple flagella at one pole will cause the flagella to bundle and rotate together to move forward, but when the direction of rotation changes for one or more of the flagella rotors in the bundle, the bundle unwinds, which causes the bacteria to tumble. Sensors that respond to environmental signals (such as light, nutrients, and chemical or osmotic gradients) regulate the switching of flagellar rotation direction, which in turn helps point the bacteria in the direction most favorable for the bacterium (discussed later).

The number and arrangement of flagella on the cell surface vary among bacterial species (Figure 11-3C), ranging from a single polar flagellum at one pole of the bacterium (monotrichous) or at both poles of the bacterium (amphitrichous) to multiple flagella arranged in tufts at one pole (lophotrichous) or evenly distributed over the entire cell surface (peritrichous). Bacteria have developed different flagellar systems to move in liquid or over surfaces. *P. aeruginosa, Vibrio cholerae,* and *Helicobacter pylori* express single or multiple flagella located on one end of the bacterium (polar flagella). This provides motility for propelling the bacteria through liquids (swimming). Polar flagella work best in dilute solutions.

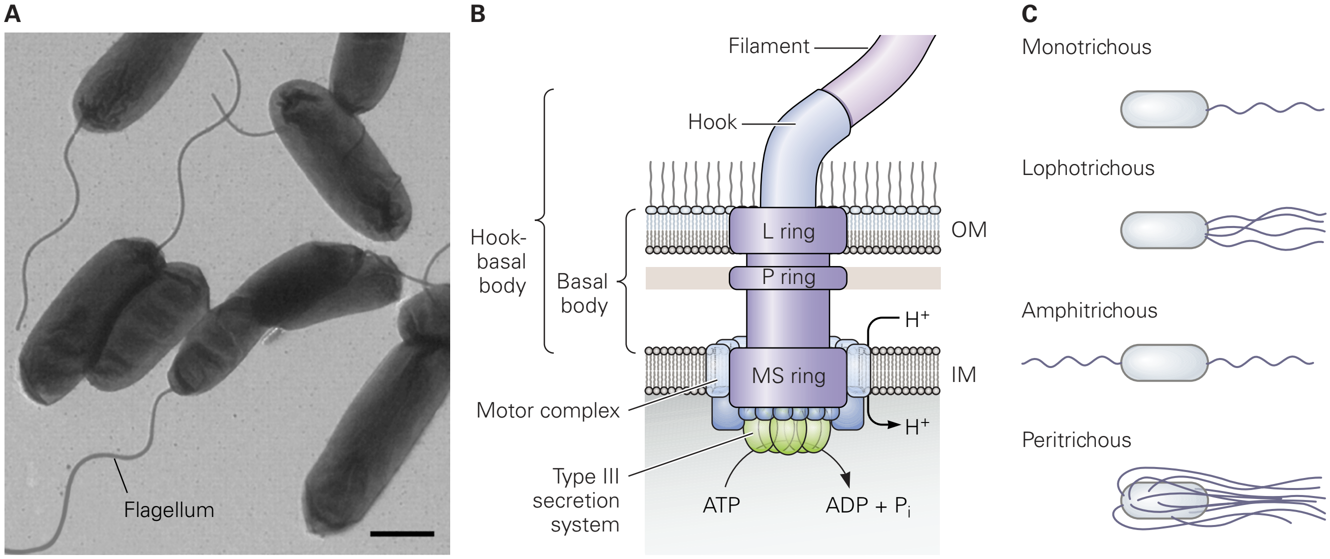


Figure 11-3. Bacterial flagellum. (A) *Vibrio cholerae* has a single polar flagellum for motility. Reprinted from Cameron DE, Urbach JM, Mekalanos JJ. 2008. *Proc Natl Acad Sci USA* **105:**8736–8741, with permission. **(B)** Shown is the basic structure of a bacterial flagellum. **(C)** Shown are the four types of flagellar organizations found in bacteria: monotrichous (with a single flagellum); lophotrichous (with multiple flagella at one pole of the cell); amphitrichous (with single flagella at opposite poles of the cell); and peritrichous (with multiple flagella projecting in different locations on the cell surface).

Other bacteria, such as *Escherichia coli, Salmonella enterica*, and *Proteus mirabilis*, express numerous lateral, constitutive flagella on their surfaces for motility in viscous media or over surfaces (swarming). A limited number of bacteria, including *Aeromonas, Azospirillum, Rhodospirillum, Vibrio parahaemolyticus, Vibrio alginolyticus, Helicobacter mustelae,* and *Plesiomonas shigelloides* species, have dual flagellar systems that are used for movement under different environmental conditions. In these systems, swimming in dilute liquid is promoted by a single, constitutively expressed polar flagellum, while swarming is enabled by numerous inducible lateral flagella, which are produced only under conditions that hamper the function of the polar flagellum. In the waterborne pathogen *V. parahaemolyticus*, a single polar flagellum is important for propulsion through water, but lateral flagella are induced after attachment to a surface, which contribute to microcolony and biofilm formation on the chitinous shells of crustaceans in the external environment. In the host environment, *Vibrio* bacteria use their polar flagellum to move through the mucous lining of the gut to gain access to the epithelial layer, where they then attach and secrete toxins.

Because the flagellum's helical structure plays an important role in its ability to move, the flagellin protein is highly conserved among different flagellated bacteria. Consequently, it should not be surprising that vertebrate innate immune systems have evolved Toll-like receptor-5 (TLR5) that specifically binds to this surface-exposed protein with similar repeating subunits (see chapter 3). Flagellins are also highly antigenic (constituting the H antigens of motile bacteria) and are often regulated in invasive pathogens such that they are not expressed once the bacterium is inside the host. In addition, flagellin biosynthesis is regulated by phase variation mechanisms that change the type of flagellin protein expressed on the bacterial surface to outfox the adaptive immune system (see chapter 7).

Another type of flagellar motility that is adapted to movement through viscous media, such as mucus and even cellular barriers, is the corkscrew motility of the spiral-shaped spirochetes. In these bacteria, two or more flagella called **endoflagella** originate from opposite poles of the bacterium and are located within the bacterium's periplasmic space. Being located in the periplasm protects endoflagella from recognition by the innate immune system's TLR5, as well as antibodies of the humoral adaptive immune system. Rotation of these endoflagella causes the bacteria to move forward in a corkscrew-like fashion. The spirochete *Treponema pallidum,* the causative agent of syphilis, is able to move readily from blood to tissue through the endothelial cells lining the blood vessels. By contrast, when placed in a drop of dilute saline solution, *T. pallidum* merely vibrates in place.

Movement by bacteria often involves the ability to sense chemical, light, oxygen, or magnetic field gradients followed by directional motility toward or away from the signal being sensed (**chemotaxis**, **phototaxis, aerotaxis,** or **magnetotaxis**, respectively). *E. coli*, the major cause of urinary tract infections, provides an example of the importance of flagella and chemotaxis. Urine is a rich source of nutrients (such as amino acids) for bacteria, so it is advantageous to a bacterium to stay in the bladder. But, since the bladder is periodically cleansed by urination, which washes unattached bacteria out of the site, the only way a bacterium can stay in the bladder is either to divide fast enough to counter dilution by new urine or to attach to the bladder mucosal cells. *E. coli* has multiple lateral flagella that allow it to swim through the mucin to the mucosa, where the bacteria can then adhere to the cells. This process involves a combination of motility and chemotaxis. The bacteria move toward **chemoattractants** (mostly amino acids in the urine) through a motility process often referred to as a **biased random walk,** which consists of a series of alternating straight runs propelling the bacteria forward and random tumbles that cause the bacteria to change direction. When the bacterium is moving in the favorable direction of the gradient, say, toward a higher concentration of chemoattractants, fewer tumbles occur. But, when the bacterium is moving in an unfavorable direction along the gradient away from the chemoattractant, then the rotation of the flagellar rotor switches from counterclockwise to clockwise, resulting in tumbling and alteration of direction. We will learn more about how the tactic sensory signals regulating motility are transmitted into a response by the bacteria in the chapter on regulatory systems (chapter 14).

Colonization of Host Surfaces

Penetrating Intact Skin

Except for a few parasitic helminths (worms), there are no known microbes that can penetrate human skin unaided by surgery, catheters, or other events that breach the skin's normal integrity. Thus, skin is the true first line of host defense a microbe must overcome. It could be argued, however, that some microbes have created their own skin trauma opportunities by colonizing biting arthropods. The bacterium that causes Lyme disease is one such pathogen. Unable to penetrate skin on its own, *Borrelia burgdorferi* enters the human or animal body through the damaged area created by the tick that carries it. Similarly, the pathogen responsible for plague, *Yersinia pestis*, penetrates and enters the host bloodstream through a flea bite. Other bacteria that can survive on skin, such as staphylococci, are in a good position to take advantage of catheters, burns, wounds, or surgical cuts as a means of bypassing the intact skin defense.

Penetrating the Mucin Layer

In many parts of the body mucosal cells are protected by **mucus,** a complex meshwork of **mucin** comprised of glycoproteins and polysaccharide. One of the roles of mucus is to act as a lubricant, but another vital function is to trap bacteria and prevent them from gaining access to mucosal epithelial cells. One of the least understood of all virulence factors is the ability of some bacteria to transit the mucin layer. Why do scientists interested in how bacteria cause intestinal or vaginal disease almost never consider the mucin layer or include it in their diagrams?

One reason for ignoring the mucin layer has been that, until recently, the procedures to fix biological samples for microscopy collapsed the mucin layers. Photographs of intestinal sections that show a layer of bacteria lying directly on the epithelial cells illustrate this artifact, yet such images have a powerful impact and have led many people to imagine that all intestinal bacteria adhere to the mucosal cells. This is almost certainly an incorrect view. Most bacteria passing through the intestine probably never come close to the mucosa because they are carried along by lumen contents or get trapped in mucin, which is then replaced so that the trapped bacteria are carried out of the site. In the respiratory tract, the ciliated cells expel bacteria caught in mucin. Bacteria that lack surface proteins or carbohydrates that bind mucin could penetrate the mucin without being trapped. The fact that most bacteria have such surface components is what makes mucin so effective in trapping them. Mucin is also highly viscous, and virtually all of the motile bacteria that cause disease in the intestinal tract are motile by means of flagella and therefore have difficulty swimming through this viscous layer.

Another reason mucin is very difficult to study is its complex structure. The mucin layer is not a uniform mat. Mucin is expelled in thick streams from **goblet cells**. Thus, the mucus is more like a field of closely spaced mucin strands than a solid mat. If so, bacteria could transit the mucin layer by moving in the spaces between mucin strands, a trick that would also help guide bacteria to the mucosal cell surface and keep them from wandering in the mucin layer parallel to the mucosal surface. Some bacteria partially digest mucin using a secreted enzyme called **mucinase**, but this is a slow and difficult process due to the complexity of mucin structure. Remember, the role of mucin is to keep bacteria away from the mucosa, so there would have been strong selection for a structure that is resistant to enzymatic digestion.

Another possibility is suggested by the observation that the **M cells** of the small intestine are the targets of most of the bacteria that cause intestinal disease. The normal role of the M cells is to sample material passing through the intestine and deliver it to the immune system associated with the gastrointestinal mucosa (see chapter 4), so M cells are not covered with a thick mucin layer. Many pathogens use the M cells as a portal through which they can transit through the mucosal layer and enter underlying tissue and blood.

There is one area of infectious disease research in which scientists have been forced to take the mucin layer into account: *H. pylori* infection. In the stomach, the mucin layer creates a buffer zone of nearly neutral pH that protects the mucosal cells from stomach acid. This zone also serves as a safe haven for *H. pylori*. If steps are taken to maintain the integrity of the mucin layer during sample preparation for microscopy, bacteria can be seen within the mucin layer as well as adhering to the mucosa. *H. pylori* has four to six flagella located at one pole of the cell. The counterclockwise rotation of the flagellar bundle, combined with the corkscrew shape of the bacterium, enables *H. pylori* to penetrate the mucin layer. The importance of the flagella for colonization of the stomach was demonstrated in studies in which a nonmotile strain was used to challenge **gnotobiotic** (**germ-free**) piglets. Only 2 of the 8 piglets were colonized by the mutant strain, while 9 of 10 piglets were colonized by the wild-type strain. Moreover, further studies showed that on those occasions when infection does occur by nonmotile strains it does not last long, and the mutant strains are cleared much sooner than wild-type strains.

Evading the Host's Innate Immunity

Secretory IgA Proteases. Bacteria attempting to colonize mucosal surfaces have to solve the problem of how to avoid being trapped in the mucin layer. The stickiness of mucin is due in part to the presence of secretory IgA (sIgA) molecules that simultaneously bind bacterial antigens via their antigen-binding sites and interact with mucin via their Fc portions. A bacterial strategy that may be designed to avoid sIgA-mediated trapping in mucin is the production of an extracellular enzyme that cleaves human sIgA in the hinge region. This cleavage would separate the part of the sIgA that binds bacteria from the part that interacts with mucin. Such enzymes are remarkably specific for sIgA and thus are known as **sIgA proteases**. Most sIgA proteases are specific for a particular human isotype, sIgA1. Another human isotype, sIgA2, which does not have the same hinge region, is not cleaved. The fact that sIgA1 is the predominant isotype in many mucosal sites may explain this specificity. The actual role of sIgA proteases in virulence is still not well understood, and there is some controversy about their importance, but the unusual specificity of these enzymes suggests that they probably play some role in colonization.

Evading Immune Recognition and Opsonization. One way to evade the host's antibody response, antigenic variation of surface structures, has already been mentioned in chapter 7. In this case, the bacterium makes the host's antibody response obsolete by providing a new antigenic type or variant of a surface protein, polysaccharide, or other molecule that is not recognized by the antibodies. Macromolecular structures, such as flagella and pili that protrude from the bacterial surface and have repeating components, are often targets for innate and adaptive immune responses. Phase and antigenic variation of the exposed surface residues is a common feature for avoiding immune recognition and clearance.

To avoid opsonization, some bacteria decorate their surfaces with proteins that attract complement factor H, instead of factor B, which inhibits complement activation. Another way to avoid the host's antibody response is for the bacterium to be mistaken as part of the host itself. As we shall see later, some bacterial capsules are composed of polysaccharides that do not trigger an antibody response because they resemble carbohydrates that are ubiquitous host tissue polysaccharides (sialic acid, hyaluronic acid). Similarly, it is conceivable that bacterial binding of lactoferrin, transferrin, and other host iron-binding proteins serves a dual function: to use them as a protective coat as well as to acquire iron from them (discussed later). Bacteria can also coat themselves with host proteins such as fibronectin. Still others may release decoy molecules into the medium that serve to sequester antibodies and immune cells through binding of the decoys instead of the bacteria.

An interesting example of this type of misdirection that might help bacteria evade the immune system is a set of bacterial proteins, including protein A of *Staphylococcus aureus* and protein G of *Streptococcus pyogenes*. These surface proteins bind the Fc portion of antibodies, thus coating the bacteria with antibodies, but in a way that does not lead to opsonization of the bacteria, because the antigen-binding portions are facing outward while the Fc portion that is usually recognized by the Fc receptor on phagocytes is hidden near the bacterial surface. This antibody coat therefore prevents recognition of the bacteria by the immune system. *S. aureus* has a number of different types of surface proteins that serve to bind to host cells as well as to evade host immune cells (Figure 11-4).

Resistance to Antibacterial Peptides. **Antibacterial peptides** can insert into bacterial membranes and create pores that allow essential cytoplasmic molecules to escape, thus killing the bacterium. Such peptides are produced in a number of body sites ranging from the base of the tongue to the crypts of the intestinal tract. Scientists first discovered antibacterial peptides in frogs when investigating why frogs seldom get wound infections despite their lack of a highly developed immune system. The answer was that they produce antibacterial peptides, called magainins, or natural antibiotics. Similar antibacterial peptides have now been found in almost every animal in which scientists have looked for them, and it has become clear that they are an important innate defense of the human body (see chapters 2 to 4). Moreover, chemokines produced by the innate immune response as attractants also have antimicrobial activity.

Human antibacterial peptides, called **defensins**, are characterized by their cationic nature and the presence of multiple cysteine residues that, through disulfide cross-linking,

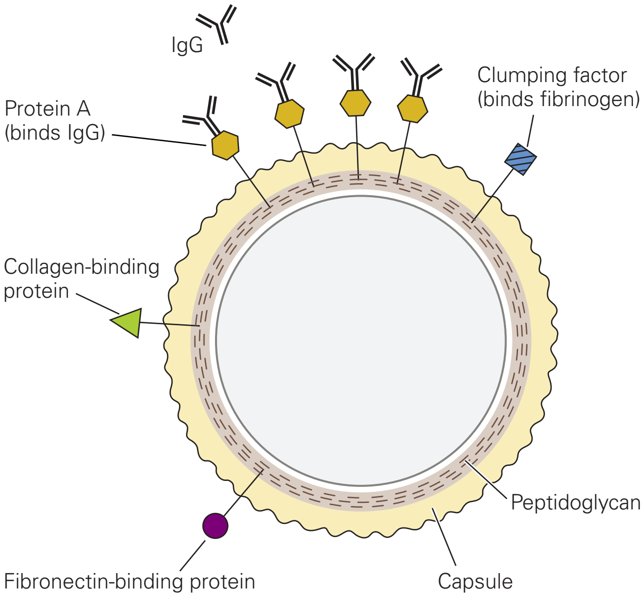


Figure 11-4. **Cell surface proteins of *Staphylococcus aureus*.** Fibronectin-binding protein (FnBP), collagen-binding protein (CBP), and fibrinogen-binding clumping factor mediate attachment to extracellular matrix proteins. Protein A provides protection from the immune system by binding the Fc portion of IgG so that the antibody cannot bind receptors on phagocytic cells. The capsule is comprised of a thick layer of polysaccharide that helps to prevent phagocytosis by phagocytic cells.

could give the peptides a circular shape. This model of their structure accords well with the hypothesis that the antibacterial peptides interact with the negatively charged bacterial cell membrane to either depolarize it or form pores in it. The difference in lipid composition between bacterial and mammalian membranes is likely a major factor in the strong activity preference that defensins show for bacterial targets instead of the host. What is still not fully understood is the mechanism by which the defensins first transit the outer membrane (in the case of Gram-negative bacteria) and peptidoglycan to reach the vulnerable cytoplasmic membrane. Presumably, defensins are small enough to diffuse through porins and through the porous net formed by the peptidoglycan. They must be able to get through somehow, because they are toxic for many types of bacteria, Gram-positive and Gram-negative alike. Nonetheless, some bacteria are resistant to defensins.

One mechanism of resistance to antibacterial peptides is production of a capsular polysaccharide layer that protects bacteria by limiting peptide diffusion to the bacterial cell surface. A second mechanism is the lipopolysaccharide (LPS) layer of Gram-negative bacteria, which is diverse in length and composition of its outer polysaccharide portion. The differences in LPS molecules among bacterial strains influence the degree of susceptibility of the bacteria to the cationic defensins. Changes in LPS length and composition can make the LPS less able to bind the defensins and thereby confer greater resistance to the defensins. For example, various Gram-negative bacteria that are resistant to the antibiotic polymyxin B have extensive modifications on their LPS that reduce the negative charge of the LPS and thereby reduce the affinity of the bacterial surface for the cationic polymyxin B. Acylation of the lipid A component of LPS and lipooligosaccharide (LOS) has also been shown to increase resistance to antimicrobial peptides. In Gram-positive bacteria, the negative charges on surface teichoic acids or phospholipid head groups are reduced through covalent modification with the positively charged amino acids D-alanine or L-lysine, respectively. Peptidases that degrade the defensins are produced by some defensin-resistant bacteria, providing yet another possible resistance mechanism. In other cases, the bacteria produce cytoplasmic proteins that counter the permeabilizing effects of the defensin channels.

The fact that bacterial resistance to defensins can occur is discouraging, because some scientists are currently developing similar peptides for use as antibiotics. The scientists had assumed that it would be difficult, if not impossible, to develop resistance to peptides that form channels in phospholipid bilayer membranes. Clearly, however, bacteria have evolved more than one strategy for dealing with the toxic-peptide problem. There are other significant challenges to developing antimicrobial peptide antibiotics, including poor availability in mammals, potential immunogenicity and cytotoxicity, degradation by host proteases, and high production costs. These problems have led some researchers to try to develop small-molecule drugs that act as mimics of antimicrobial peptides.

Nutrient and Iron Acquisition Mechanisms

Nutrient Acquisition. Pathogens must find nutrients and appropriate environmental conditions to grow and establish an infection. However, not all nutrients and elements (i.e., iron) are in adequate supply in all tissues of the host body, and their availability can influence the establishment of an infection at a particular site versus another and thereby impact the virulence of the bacterium. For example, *Brucella* is a Gram-negative pathogen that causes the zoonotic disease *brucellosis* in domestic animals such as cows, sheep, goats, and horses, and sometimes humans. Different *Brucella* strains have host specificity. The bovine-specific *Brucella abortus* grows very slowly in most tissues of a cow's body, except in the placenta of pregnant animals, where it grows very rapidly and results in abortion. This propensity to proliferate in the placenta is due to the presence of high concentrations of erythritol in this tissue, a sugar that the bacterium can easily metabolize.

Many pathogens express degradative enzymes, such as proteases, glycosidases, nucleases, and lipases, which they display on their surfaces or secrete into the medium. These enzymes break host macromolecules down to their building blocks, such as amino acids, sugars, lipids, and other nutrient components, which are then absorbed or transported into the bacterium. Bacterial phospholipases, for instance, cleave membrane phospholipids, which in turn lyse host cells, releasing the cellular contents for utilization as food by the bacteria. Bacterial induction of host inflammatory responses, likewise, causes tissue damage that releases cellular contents, which can be exploited by the bacteria for food. As discussed further elsewhere in this chapter, these degradative enzymes also serve other functions, such as easing the way for pathogens to move through viscous media by degrading mucin to allow access to the epithelial layer, degrading extracellular matrix for movement through tissues, or degrading DNA and RNA in pus that is released from neutrophils or from damaged host cells.

Iron Acquisition. Most articles on iron acquisition by bacteria start with the statement that iron is essential for the growth of all bacteria. It is now becoming apparent that this statement is not universally true. There are some bacteria that solve their iron acquisition problem by not using iron at all. For example, the causative agent of Lyme disease, *B. burgdorferi*, uses this iron abstinence strategy. It lacks all the iron-dependent cofactors and enzymes and uses manganese (Mn^{2+}) instead of iron to do the chemistry it needs for survival and replication. This interesting exception notwithstanding, most pathogenic bacteria do require iron and have to cope with the fact that free iron concentrations in hosts are extremely low.

Estimates of the free iron concentration at different sites in the human body can range from 10^{-18} to 10^{-9} M, depending on the body site. These low concentrations of free iron are due to the action of host iron-binding and transport proteins, such as **lactoferrin** (protein secreted at mucosal surfaces and in milk, used for chelating iron at mucosal surfaces), **transferrin** (made in the liver, the serum protein responsible for transport of iron in blood and tissue), **ferritin** (the major protein used for intracellular iron storage), and **heme** (in humans, ~70% of total body iron is found in **hemoglobin**), that bind most of the available iron. To survive in the body, bacteria must have some mechanism for acquiring this sequestered iron and, consequently, Gram-negative and Gram-positive bacteria have developed a number of possible ways to gain access to this vital element (Figure 11-5).

The best-studied type of bacterial iron acquisition mechanism is the production of **siderophores**. Siderophores are low-molecular-weight compounds that chelate iron with high affinity, which allows them to bind iron when it is present in extremely low concentrations, such as that found in the host environment. There are two main classes of siderophores, **catechols** and **hydroxamates**, which differ in structure (Figure 11-6). Both, however, have the same property, namely, that they form tight iron-chelated complexes with high affinity.

Siderophores are excreted into the medium by the bacteria. The siderophores bind to free iron in the medium, and then the iron-siderophore complex is taken back up into the bacteria by special siderophore receptors on the bacterial surface, which differ between Gram-negative and Gram-positive bacteria due to the presence of the outer membrane in Gram-negative bacteria (see Figure 11-5). In Gram-negative bacteria, periplasmic protein complexes that energize transport using the proton motive force (PMF) across the cytoplasmic membrane mediate siderophore-iron transport through a receptor pore across the outer membrane. Siderophore-iron complexes released into the periplasm of Gram-negative bacteria or at the extracellular surface of Gram-positive cells interact with a substrate binding protein that delivers the siderophore-iron complex to an ABC-type transporter that then energizes transport into the cytoplasm by using ATP hydrolysis. The internalized iron-siderophore complex is cleaved to release the iron molecules inside the bacteria.

Some bacteria not only produce their own siderophores, but also produce receptors capable of binding siderophores produced by other bacteria, the bacterial equivalent of freeloading. This property is thought to contribute to the synergism that sometimes occurs during coinfections, where one bacterium that does not make siderophores but does make siderophore receptors benefits from the presence of another bacterium that makes siderophores. Although siderophore-based iron acquisition has been shown to contribute to virulence of many bacterial pathogens, mutations that eliminate siderophore production or uptake by a bacterial pathogen do not always significantly decrease virulence. This finding could be explained by the fact that bacteria often have more than one iron-sequestering system, and a mutant deficient in one system may still be able to survive by relying on the remaining system. Nonetheless, it is also possible that siderophore-based iron-acquisition systems are adapted mainly for survival of the bacteria outside the body, in soil or water, whereas other strategies of iron acquisition are more important in the human body.

Bacteria have access to free iron in soil or water, whereas in the human body virtually all of the iron is already bound to proteins such as hemoglobin, transferrin, or lactoferrin. A number of pathogenic bacteria are able to use these iron-binding proteins as sources of iron (Figure 11-5). In Gram-negative bacteria with these capabilities, specific outer-membrane receptors bind the iron-containing host molecule as part of the iron-acquisition process. How they remove the iron from the host proteins is still unclear for many of these systems, but the most common mechanism appears to be direct binding and processing of the iron-containing protein and release of the iron into the periplasm, followed by transport of the iron into the cell. As more and more pathogens are studied, it is becoming apparent that acquisition of iron from transferrin or lactoferrin, once

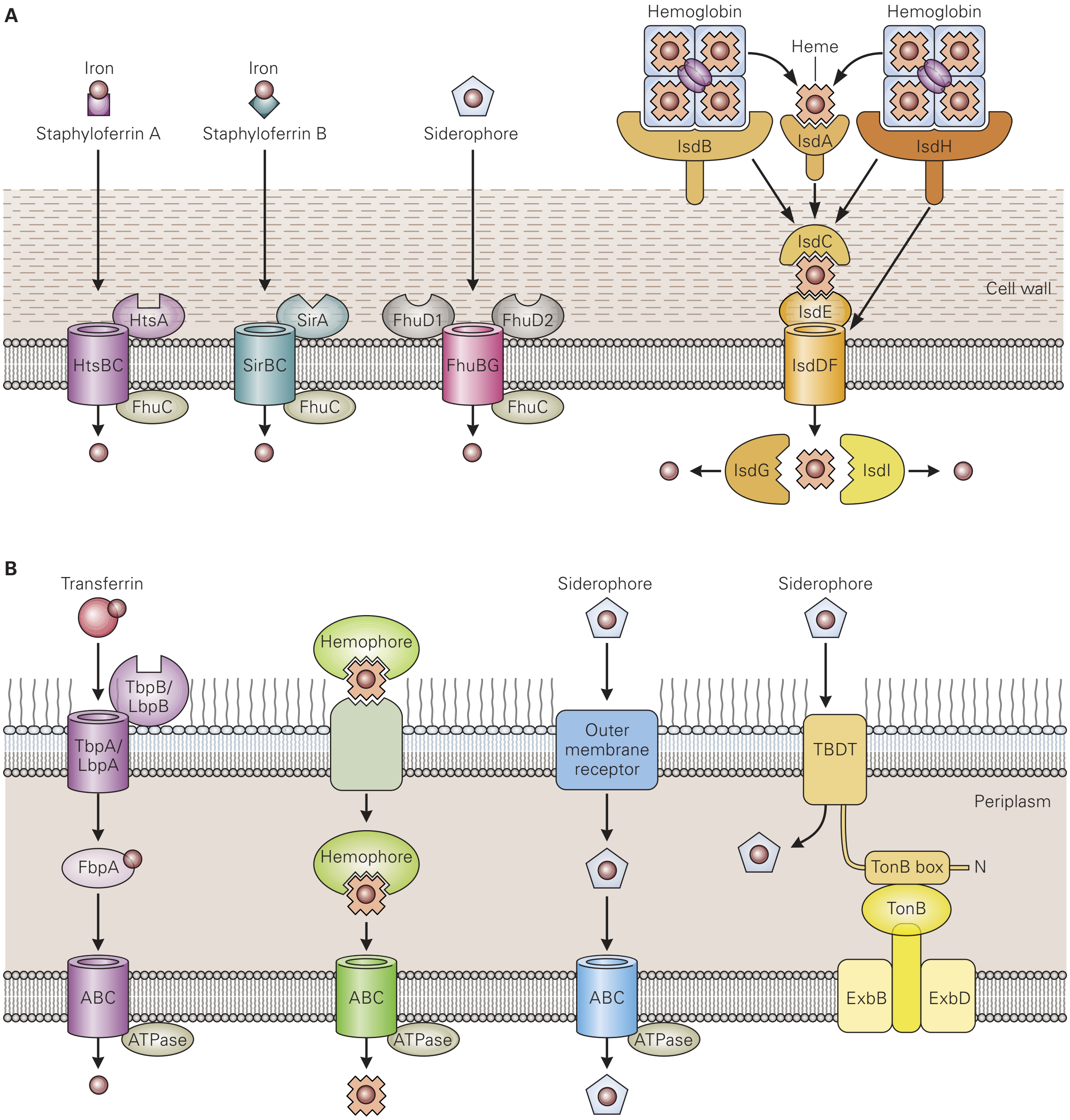
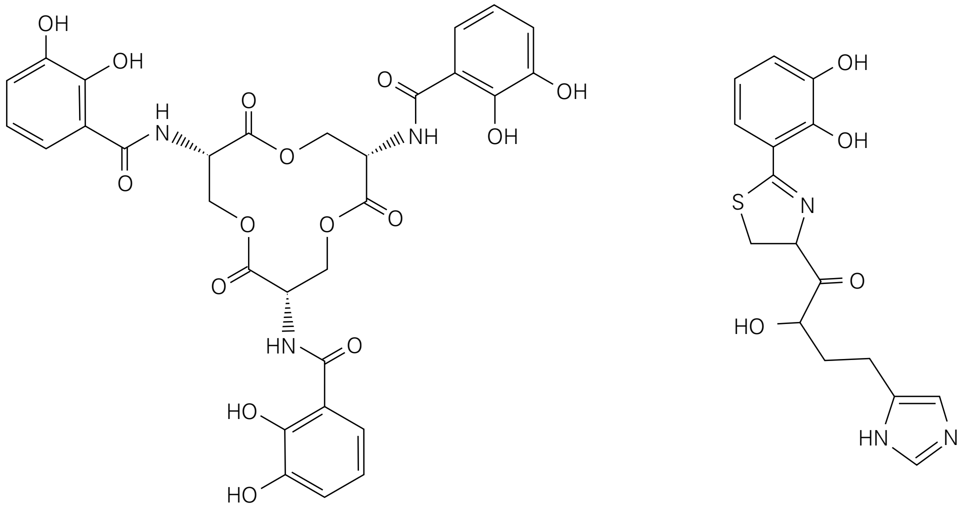

A
Iron
Staphyloferrin A
Iron
Staphyloferrin B
Siderophore
Hemoglobin
Heme
Hemoglobin
IsdB
IsdA
IsdH
IsdC
IsdE
Cell wall
HtsA
SirA
FhuD1
FhuD2
HtsBC
SirBC
FhuBG
IsdDF
FhuC
FhuC
FhuC
IsdG
IsdI
B
Transferrin
Siderophore
Siderophore
Hemophore
TbpB/
LbpB
TbpA/
LbpA
Outer
membrane
receptor
TBDT
Periplasm
FbpA
Hemophore
TonB box
N
TonB
ABC
ABC
ABC
ATPase
ATPase
ATPase
ExbB
ExbD

Figure 11-5. Iron-acquisition mechanisms. (A) In Gram-positive bacteria, which lack an outer membrane, uptake of iron from transferrin/lactoferrin, siderophores, or heme involves a bacterial membrane-anchored Fe-binding protein and a membrane-associated ABC transporter system. *Staphylococcus aureus* has two iron-binding siderophores, staphyloferrin A and B, which are recognized by their cognate lipoprotein surface receptors, HtsA and SirA, and the heterodimeric permeases, HtsBC and SirBC, respectively. Import of ferric-bound siderophores by these iron uptake systems is driven by ATP hydrolysis through coupling to the ATPase FhuC. *S. aureus* can use hydroxamate-type siderophores exogenously produced by other bacteria to take up iron through the ferric hydroxamate (Fhu) ABC transporter system. *S. aureus* can also scavenge iron-bound heme and hemoglobin through a series of iron-regulated surface determinants (Isd) proteins: IsdH, IsdB, IsdA, and IsdC, which are covalently linked to the cell wall; the periplasmic receptor IsdE; and the heterodimeric permease complex IsdDF, which transports the ferric-bound heme into the cytoplasm, where the heme-degrading enzymes IsdG and IsdI act to release the iron. **(B)** In Gram-negative bacteria, there are numerous iron uptake pathways. All of these uptake pathways include an iron uptake mechanism, via transferrin/lactoferrin, siderophores or heme, and require an outer membrane receptor, a periplasmic binding protein, and an inner-membrane ABC transporter system. For example, pathogenic bacteria from the *Neisseriaceae* and *Pasteurellaceae* families have a host transferrin-specific iron uptake system mediated by the bacterial receptor complex of the transferrin-binding proteins A and B (TbpA and TbpB). TbpB is a lipoprotein that binds to Fe-bound host transferrin, and TbpA is a TonB-dependent transporter (TBDT) that binds and delivers both Fe-bound and apo forms of transferrin into the periplasm. Similarly, transport of Fe-bound siderophores or Fe-bound heme-hemophore complexes across the outer membrane into the periplasm is mediated by a TBDT, which derives energy from the inner membrane proton-motive force, which requires the action of the energy-transducing TonB system, comprised of TonB, ExbB, and ExbD. Movement through TBDT requires interaction of the TonB box at the N-terminus of TBDT with TonB, with the energy for conformational changes provided by ExbB and ExbD. In the periplasm, a periplasmic Fe-binding protein (FbpA) serves as a receptor to bind and mediate transport of the Fe-transferrin, Fe-siderophore, or Fe-heme into the cytoplasm via the ABC transporter in an ATP-driven manner. Cytoplasmic proteins then facilitate release of the iron.



Enterobactin

Anguibactin

Figure 11-6. Iron acquisition by bacterial siderophores. Siderophores differ considerably in structure (compare the catechol enterobactin from *E. coli* to the hydroxamate anguibactin from *Vibrio anguillarum*), but they are also similar in that they are basically just iron chelators with a very high affinity for iron. The siderophore sequesters the iron away from the host iron-binding proteins, and the iron-siderophore complex is taken back up by the bacteria via receptors and released into the bacterial cytoplasm.

thought to be an unusual mechanism of iron acquisition, is much more common than previously suspected. Bacteria can also produce proteases that degrade transferrin or lactoferrin to release iron. Acquisition of iron directly from hemoglobin and heme has also been detected in Gram-positive bacteria, such as *S. aureus*, which possesses systems that take up heme into the bacterial cells, where heme oxygenases in the cytoplasm strip off the bound iron.

Another possible iron acquisition strategy of bacteria is production of toxic factors, such as hemolysins and other pore-forming cytolysins, that kill host cells. As will be seen in the next chapter, some bacterial toxins are iron-regulated and produced only when iron levels are low. Because these toxic proteins kill host cells, they might be part of an iron acquisition strategy in which host cells are killed by the toxin to release their iron stores (primarily ferritin- or heme-bound iron), which can then be acquired by the bacteria via ferritin- or heme-binding proteins. Of course, it probably should be noted that low levels of iron as a signal for regulation of virulence gene expression is not necessarily directly linked to iron acquisition; it could merely be a signal that the bacterium uses to sense that it is in a host environment.

Adherence

Role of Adherence. One of the dogmas of bacterial pathogenesis research is that adherence is an essential first step in the disease process. As we have just seen, however, bacteria such as *H. pylori* can stay in a particular site by being trapped in the mucin layer. These bacteria need not have special structures for adherence. Moreover, there is some evidence that certain strains of *E. coli*—the most common cause of urinary tract infections—divide rapidly enough in urine to stay in the bladder without adhering. Nevertheless, although adherence may not be critical for all pathogens, it is an essential first step for colonization of most bacteria. In the mouth, small intestine, and bladder, mucosal surfaces are constantly washed by fluids, which keep down the number of bacteria present in the site. In such locations, bacteria capable of adhering to mucosal cells have a notable advantage. Even in other slower-moving areas, such as the colon and vaginal tract, Brownian motion can move a bacterium that has made contact with a mucosal cell away from the surface of the cell. Virtually all known bacterial pathogens—and a lot of nonpathogenic bacteria, for that matter—have ways of attaching themselves firmly to host cells as well as each other to form microcolonies or biofilms. The main strategies bacteria use to attach themselves to host cells are illustrated in Figure 11-7.

Pili and Fimbriae. The best understood mechanism of adherence is attachment mediated by rod-shaped, filamentous protein structures called pili (or fimbriae). Proteinaceous pili (or fimbriae) are surface **adhesins** most often found on Gram-negative bacteria, but also on some Gram-positive bacteria. The filaments differ in thickness and length. The term **fimbriae** (singular **fimbria**) has been used to designate shorter, thinner structures, whereas **pili** (singular **pilus**) is the term used to designate longer, thicker surface structures. However, this convention is not always

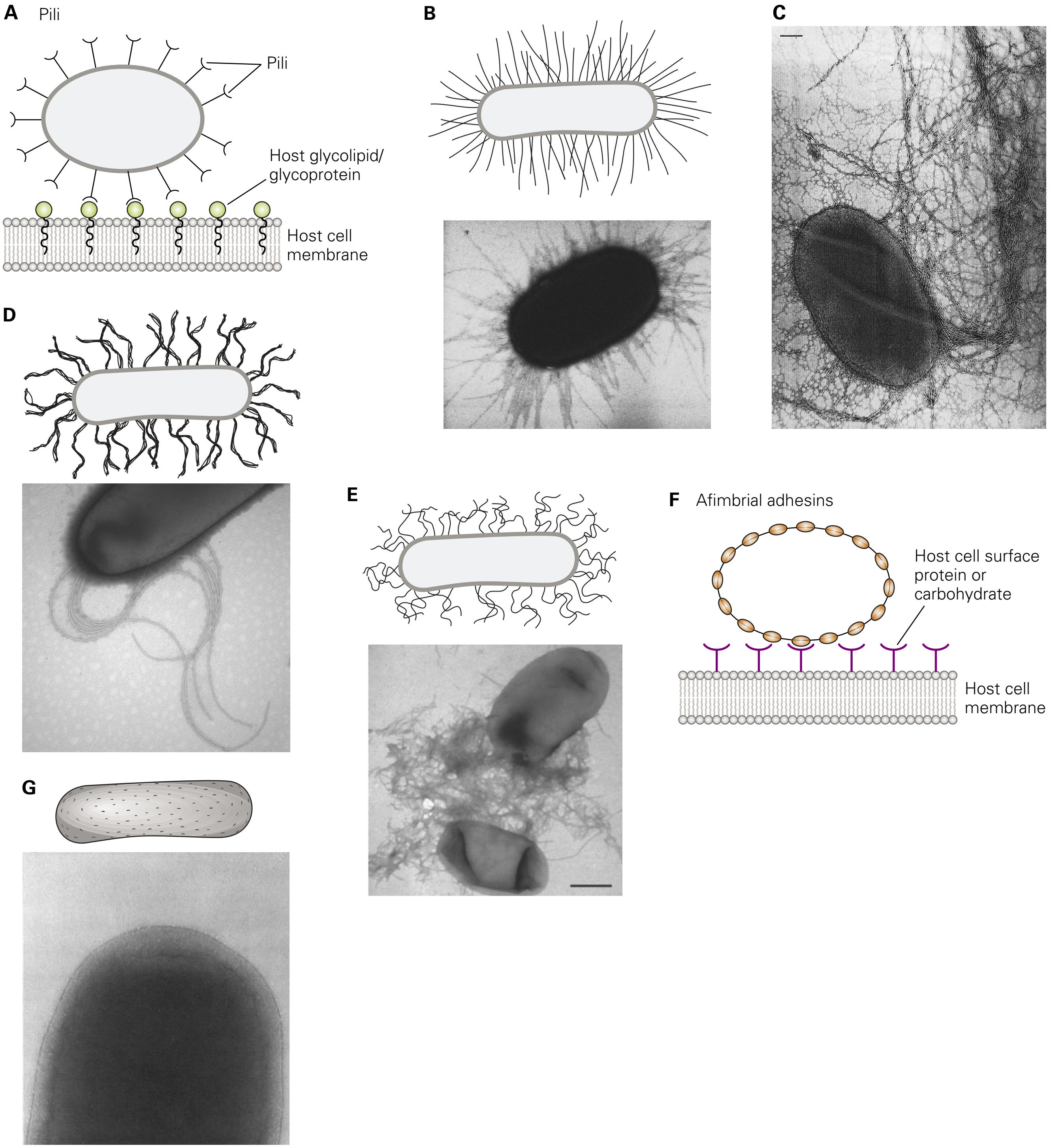


Figure 11-7. Types of Gram-negative bacterial adherence mechanisms. (**A**) Shown is the mechanism by which pili, the rod-shaped protein structures that extend from the bacterial surface, bind to host cell surface molecules, usually carbohydrates. The tip structure is magnified in this drawing to emphasize its presence. In reality, the tip structure is much smaller than this figure would indicate. (**B**) Type 1 pili (and longer P pili) are rigid, long, thin filaments that protrude from the surface of Gram-negative bacteria. Shown is an electron micrograph of *E. coli* type 1 pili. Reprinted from Gross L. 2006. *PLoS Biol* **4:**e314 (image: Manu Forero), with permission. **(C)** Type 2 pili from *Burkholderia cepacia* form large cable-like intertwined structures that contribute to biofilm formation in the lungs of cystic fibrosis patients. Reprinted from Sajjan US, Sun L, Goldstein R, Forstner JF. 1995. *J Bacteriol* **177:**1030–1038, with permission. (**D**) Type 4 bundle-forming pili form flexible ropelike structures made of many individual threads intertwined into bundles and tangled with each other. In *Pseudomonas aeruginosa*, type 4 pili mediate twitching motility. Micrograph courtesy of Louisa Howard, Dartmouth Electron Microscope Facility. (**E**) Type 5 pili (also called curli pili) are curved or curled thin, intertwined filaments that form long, amyloid-like coiled structures that contribute to aggregation, microcolony formation, and/or biofilm formation. Shown is an electron micrograph of *Salmonella* Typhimurium curli pili. Reprinted from Evans ML, Chapman MR. 2014. *Biochim Biophys Acta* **1843:**1551–1558, with permission. (**F**) Mechanism by which afimbrial adhesins, bacterial surface proteins that are not organized in a rodlike structure, but instead are close to cell surfaces, mediate tight binding between bacteria and host cell. **(G)** Afimbrial adhesins are embedded in the surface of bacteria. Shown is an electron micrograph of the surface of an *E. coli* cell. The afimbrial adhesion is notable because there are no fimbrial structures visible, but the bacteria still sticks to cells. Reprinted from Le Bouguenec C, Garcia MI, Ouin V, Desperrier JM, Gounon P, Labigne A. 1993. *Infect Immun* **61:**5106–5114, with permission.

observed and the two terms are often used interchangeably. Still other researchers reserve the term pilus for the sexual appendage required for bacterial conjugation and use the term fimbriae for all other adhesins. In this book, pilus/pili will be used as the default terms unless researchers in a particular field have made a point of using fimbria/fimbriae.

There are many different types of pili that have been characterized in both Gram-negative and Gram-positive bacteria, and various forms are involved in attachment to diverse types of matrices and surfaces of host cells or inanimate objects, as well as to each other in bacterial cell aggregation and microcolony formation or to each other and other types of microbial cells in biofilms. The number of pili on a bacterial cell can vary from just a few pili to several hundred and, in some cases, up to a thousand or more. In Figure 11-7, pili are shown evenly distributed over the surface of the bacteria, but in some cases they may be located preferentially at one pole of the bacterial surface.

A pilus is a rod-shaped chain of polymerized subunits of the protein called **pilin** that extends outward from the bacterial surface. The major pilin protein subunits are usually about 20 kDa in size and are packed in an ordered helical array to form a flexible cylindrical structure of varying length, ranging from less than 0.5 µm up to 10 µm or more, depending on the bacterial species and type of pili. For instance, over 30 different types of pili have been identified for different *E. coli* strains, many of which contribute to host cell- or tissue-specific interactions, called **tropism**.

Pili act to establish contact between the bacterial surface and the surface of the host cell. The protein(s) at the tip of the pilus is the actual adhesin that attaches to a matrix component or to a molecule on the host cell surface. Host cell receptors for pili are commonly carbohydrate residues of glycoproteins or glycolipids. These host molecules are often involved in targeting of the host cell to its ultimate site, mediating cell-cell contact, or serving as part of the host cell's signal transduction mechanisms. Because of their importance for host function, the receptors on host cells recognized by pili are usually strongly conserved evolutionarily, making it easier for different types of bacteria to subvert them for their own use as general attachment sites.

Within a host, adherence between bacteria and other surfaces such as host cells can occur through multiple interaction mechanisms involving selective or nonselective binding of the surface components on the two cells. Lectins are carbohydrate-binding proteins on host cell membranes that bind with high specificity to certain sugar groups on glycoproteins or glycolipids present on certain bacteria or other host cells. As such, they often mediate microbial binding to host immune cells. For example, mannose-binding lectin (MBL) of the innate immune system binds to mannose groups on bacterial cell surfaces, which triggers the complement cascade. In contrast, the minor tip adhesin proteins of pili/fimbriae can act like lectins by binding to carbohydrate groups on glycoproteins or glycolipids that are present on the host cell surface or on other bacterial cell surfaces. In both cases, the addition of free sugar (such as mannose) to the medium can disrupt the binding interactions between the cells.

Binding of a pilus to its host-cell target is quite specific. This specificity is important because the availability of suitable receptors will often determine what body site is infected by the bacterium. The carbohydrate residues bound by the pilus can often be identified by simple competition assays. Addition of the free carbohydrate in excess will saturate the binding sites on the pilus and prevent binding to cultured host cells.

In many cases, the specific binding between the pilus tip and the host cell carbohydrate is mediated by a specialized tip structure that consists of several pilin proteins distinct from the shaft pilin. In other cases, binding of the tip appears to be mediated by the pilin protein itself. It is not always easy to determine whether a pilus has a specialized tip structure because the main structural pilin subunit accounts for over 99% of the protein in the pilus. Thus, minor pilin proteins that form a specialized tip structure may be missed in the initial biochemical analysis of the pilus composition. Now that DNA sequencing has become so prevalent, genes coding for minor proteins are often detected as unannotated **open reading frames (ORFs)** located in operons containing the main pilin subunit gene and genes that encode proteins that help assemble the pilus.

Why do bacteria use such long, fragile structures for adherence? One plausible explanation is that since the surfaces of bacteria and host cells are both negatively charged, pili allow the bacteria to bind host cells without getting close enough for electrostatic repulsion to prevent attachment. Another possibility is that pili allow the bacteria to make an initial loose contact with a host cell surface, which then triggers production of surface proteins needed to mediate tighter binding. Indeed, many pathogenic bacteria appear to use a two-step process for attachment, in which a loose association is first made using pili. This is then followed by depolymerization of the pili and a tighter binding involving other surface proteins that bring the bacterial and host cell surfaces closer together. Some pathogens that use pili as adhesins are severely attenuated when the pili are absent, and in those cases pili are considered critical virulence factors. Finally, some pili described in the following sections are retractable and provide a mechanism for the "twitching" motility of certain bacteria on certain surfaces.

Pili (Fimbriae) of Gram-Negative Bacteria. The major types of pili found in Gram-negative bacteria are grouped based on their structure, how they are made, and the nature of the adhesin (ligand) and cellular receptor they use. Bacteria can have many different types of pili, not all of which are expressed at the same time. For example, *E. coli* can have as many as 12 different types of pili. In a genome, separate gene clusters, often called *pil* or *fim* gene operons, encode the assembly machinery (apparatus, chaperones) and the pilus components (pilin and tip proteins) that are distinct for each type of pilus and are under the control of different regulatory systems.

The best-characterized pili in Gram-negative bacteria are the type 1 and Pap (or P) pili of *E. coli*, encoded by the *fim* and *pap* gene operons, respectively (sometimes called *pil* genes in other bacteria). Type 1 pili are rigid rodlike structures that are assembled through a chaperone-usher system (discussed more later) and cause red blood cells (RBCs) to undergo **hemagglutination** (aggregation or clumping of the RBCs) in a mannose-sensitive manner, that is, addition of mannose blocks the binding of the bacteria to the RBCs and prevents hemagglutination. In this case, the *E. coli* type 1 fimbrial adhesin FimH has an N-terminal carbohydrate-binding domain that recognizes D-mannose groups on surface molecules. Addition of free mannose into the medium blocks FimH binding to the surface.

Type 2 pili are similar to type 1 pili, but do not induce hemagglutination. *Burkholderia cenocepaciae* strains isolated from patients with cystic fibrosis adhere to mucus glycoproteins in the lungs and oral epithelial cells via type 2 pili. These peritrichous pili form large cable-like structures made of very long (2–4 μm) intertwined fibers of the major cable (Cbl) pilin protein, encoded by the *cblA* gene. The cable fibers often tether other adjacent bacteria into mat-like biofilms in the lungs of cystic fibrosis patients. The minor pilin adhesin protein can adhere to mucin and to an epithelial cell receptor, but unlike other adhesin proteins it does not appear to be located only at the tip of the cable fibers, but also dispersed along the length of the cable fiber.

Type 3 pili are more flexible than type 1 or type 2 pili and are resistant to mannose. They are commonly found in Enterobacteriaceae, including *E. coli, Klebsiella pneumoniae*, and *Salmonella* species. Type 3 pili are not found in all strains of *E. coli*, but they are found in most clinical isolates from catheter-associated urinary tract infections. As with *E. coli*, type 1 and type 3 fimbriae of *K. pneumoniae* are produced by clinical isolates, where type 1 pili mediate adherence to epithelial cells in the urogenital tract and type 3 fimbriae mediate adherence to endothelial and bladder epithelial cells. Importantly, type 3 fimbriae play a major role in biofilm formation through attachment to abiotic surfaces such as indwelling devices like catheters, which contributes to their role in hospital-acquired infections. The requirement of pili for urinary tract infections by *E. coli* and structural knowledge of pilus biogenesis has led to the design and testing of new classes of potential antibiotics, call **pilicides.**

Type 4 pili (often called fimbriae) have an N-methylphenylalanine at the N terminus of the major pilin subunit. They are flexible and often appear as bundles found at the poles of a wide variety of Gram-negative pathogens, including *Neisseria* species, *Pasteurella multocida, Dichelobacter nodosus, Moraxella bovis*, *Aggregatibacter actinomycetemcomitans*, and *P. aeruginosa*, where they mediate adhesion and twitching motility by attaching to the surface through the pilin tip and then retracting the pili back through the outer membrane into the periplasm. This action pulls the bacterium toward the attachment site in a jerky motion, similar to the action of a grappling hook.

Type 5 (curli) pili are much thinner than mannose-sensitive type 1 fimbriae and appear in electron micrographs as tangled masses of fibers 4–6 nm in width and 2–4 μm in length. Curli pili are composed of a β-sheet-containing subunit protein that forms long, amyloid-like coiled structures that nucleate and polymerize on cell surfaces. They are often found in clinical strains of *E. coli* and *Salmonella* species, where they adhere to extracellular matrix proteins and contribute to aggregation, microcolony, and biofilm formation. Curli pili enable adherence to surfaces such as Teflon and stainless steel, which can be problematic, since adhesion can result in biofilm formation in hospital settings and contamination in food processing plants. Like other amyloid fibers, curli pili are resistant to protease degradation or dissociation with 1% sodium dodecyl sulfate. Colonies of curli-producing bacteria stain red when grown on plates containing the dye Congo red and fluoresce when grown on plates containing thioflavin T; both properties are used as diagnostic indicators.

The assembly of pili by Gram-negative bacteria occurs via some variation of a **chaperone-usher system**, a complex process that requires participation of a large number of auxiliary proteins. Much has been learned about pilus assembly in recent years, leading to more and more complex models. Despite the diversity in pilus structure and biogenesis, the shaft of the pilus is formed by noncovalent polymerization of the major pilin subunit protein with other minor pilins (tip proteins) added, which often serve as host cell adhesins. In Figure 11-8, we provide a simplified model of the process for the common type 1 pili of *E. coli*. An analogous set of proteins are involved in assembly of the P pili of *E. coli*. The first step is secretion of pilin (FimA in type 1 pili, PapA in P pili) and specialized tip proteins (FimH, FimG, FimF in type 1 pili; PapE, PapF, PapG, PapK in P pili) across the inner membrane and into the periplasmic space. In this case, secretion of pilin and the tip proteins into the periplasm appears to be mediated by the normal SecAB protein secretion system of the bacteria (see chapter 13). But for some other kinds of pili, a specialized pilus-specific secretion system is used. Thus, proteins that are secreted through the normal secretion machinery have an amino-terminal signal sequence that is proteolytically removed during processing. Pilin subunits of some pili still have a methionine residue at their amino terminus, indicating that normal proteolytic processing did not occur during export by the general bacterial Sec secretion system. Still

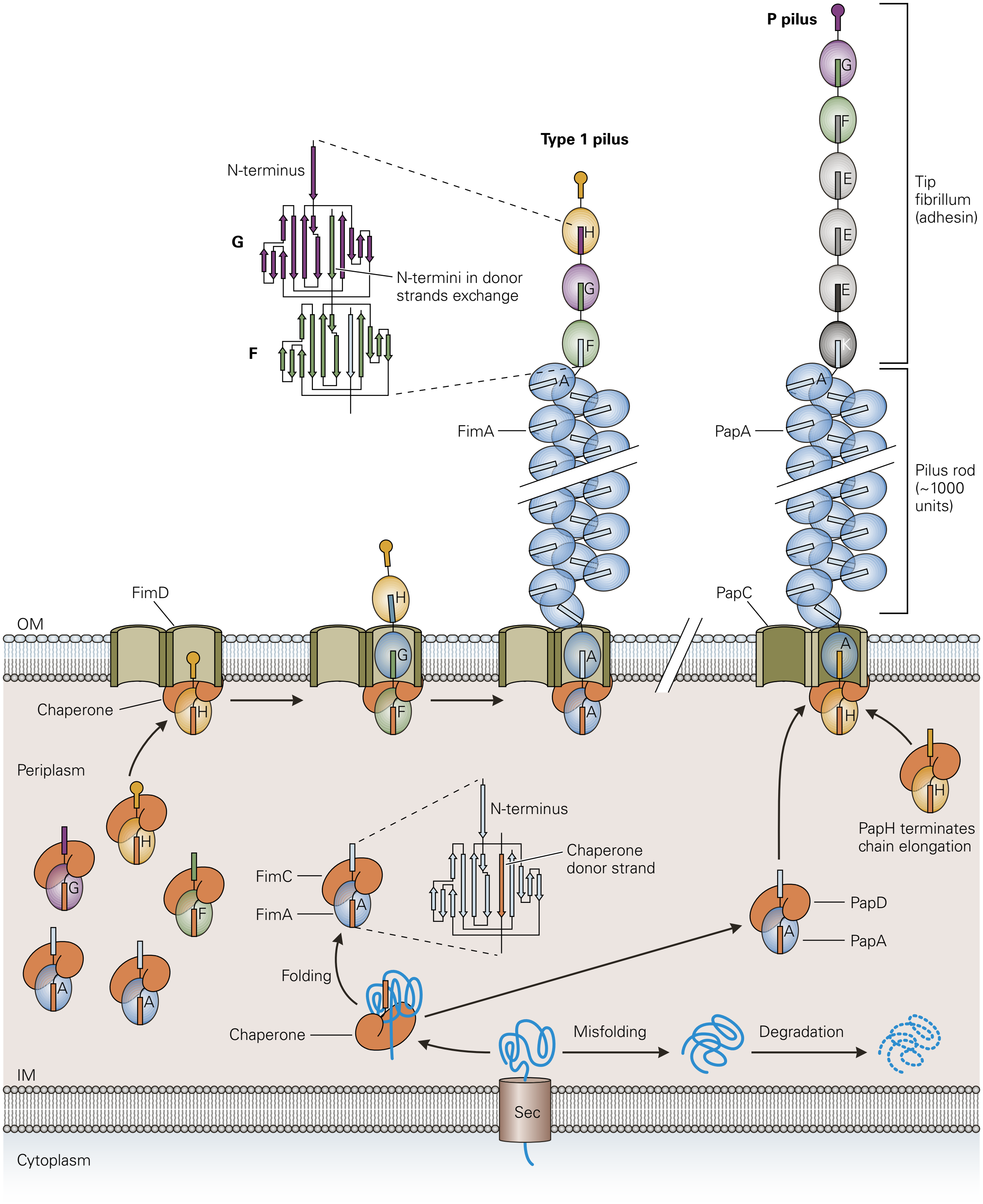

P pilus
G
F
E
E
E
K
Tip fibrillum (adhesin)
Type 1 pilus
N-terminus
G
N-termini in donor strands exchange
F
H
G
F
A
FimA
PapA
A
Pilus rod (~1000 units)
FimD
PapC
OM
Chaperone
H
G
F
A
A
A
H
Periplasm
N-terminus
Chaperone donor strand
PapH terminates chain elongation
H
FimC
FimA
A
G
H
F
A
A
PapD
PapA
A
Folding
Chaperone
Misfolding
Degradation
IM
Sec
Cytoplasm

Figure 11-8. **Model for assembly of type 1 and P pili with adhesive tip structures in Gram-negative bacteria.** The assembly of a type 1 pilus is shown on the left and that of a P pilus on the right. The size of the tip structure is exaggerated to emphasize its location and different structure compared to the pilus shaft itself. The adhesive tip components of the type 1 pilus (FimH, FimG, and FimF) are assembled first and then the main pilus subunit (FimA) is assembled to form the pilus rod. Each subunit donates an N-terminal strand to the prior subunit during assembly of the pilus. Chaperones (FimC) prevent aggregation and misfolding of the pilin subunits in the periplasm and deliver the subunits to the dimeric FimD usher protein embedded in the outer membrane. Note that the chaperone donates a strand to the subunit to stabilize the structure and prevent assembly in the periplasm. The assembly process of type P pili is similar. Note that the subunits for each type of pili have slightly different order and designations (Fim versus Pap). Adapted from Li H, Thanassi DG. 2009. *Curr Opin Microbiol* **12:**326–332, with permission.

other types of pilin have a methyl group attached to their amino-terminal amino acid (often a modified phenylalanine residue) after secretion and processing.

Once in the periplasm, special proteins called **chaperones** (FimC in type 1 pili, PapD in P pili) prevent the pilin subunits from folding into their final configuration and aggregating in the periplasm. In support of this role, pilin subunits aggregate in the periplasm of bacterial mutants that do not produce the chaperone, setting off a drastic stress response in the bacteria. The chaperones guide the bound pilin subunits to an outer membrane **usher** protein dimer (FimD in type 1 pili, PapC in P pili), where assembly and export of the pilin structure begins. The adhesive tip structure is assembled and extruded first, then the shaft of the pilus is made by sequential addition of pilin subunits to the base of the pilus, pushing the already assembled tip portions outward from the bacterial surface. This handoff of subunits during pilus biogenesis occurs by a mechanism of complementary donor strand exchange. Finally, a termination protein (FimH in type 1 pili, PapH in type P pili) signals the end of the extrusion process and presumably stabilizes the resulting pilus on the cell surface. How the pilus is actually stabilized in the outer membrane, and how the large pore of the usher protein functions, are areas of active research.

Type 4 pili are assembled through a two-step process involving transport first to the periplasm by a modified Sec- or TAT-dependent system, followed by transport through the outer membrane onto the surface by a type 2 secretion system (T2SS, see chapter 13). By analogy, *E. coli* type 5 (curli) pili are assembled by first transporting subunits to the periplasm by the general SecAB-YEG protein exporter, followed by secretion to the surface by proteins encoded by two operons (Figure 11-9). The *csgBAC* operon encodes the major structural protein CsgA, the nucleator protein CsgB (which induces the amyloid fiber formation of CsgA), and the periplasmic chaperone CsgC (which prevents premature amyloid fiber formation). The *csgDEFG* operon encodes the CsgD positive transcriptional regulator of the *csgBAC*, two chaperone proteins CsgE and CsgF, and integral membrane protein CsgG that forms a nonameric channel in the outer membrane. Homologous operons are found in *Salmonella* species: *agfBAC* and *agfDEFG*, where the expressed proteins form fibers called Tafi (for thin aggregative fimbriae).

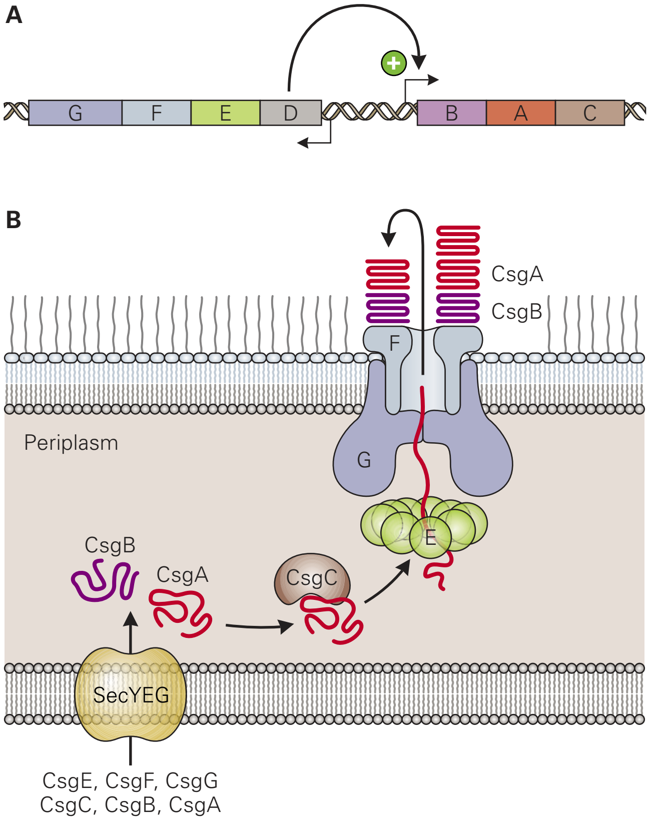


Figure 11-9. Models of curli pilus regulation and assembly. (A) A schematic diagram of the two operons *csgBAC* and *csgDEFG* involved in curli-pili biogenesis. CsgD is a positive transcriptional regulator of the *csgBAC* operon. **(B)** Mechanism for curli assembly on the external surface of the outer membrane. All of the proteins encoded by the *csg* operons, except CsgD, have secretion signal sequences for delivery to the periplasm. CsgA is the major pilin structural protein that is secreted by CsgG with the help of CsgE and CsgF across the outer membrane. CsgB is also secreted and serves to trigger the nucleation of CsgA into the curli fibers. CsgC acts as a periplasmic chaperone that inhibits premature CsgA polymerization in the periplasm.

Pili (Fimbriae) of Gram-Positive Bacteria. Most studies of pili have focused on Gram-negative bacteria. In the past decade, much more information has been obtained about the adhesins of Gram-positive bacteria. Indeed, pili have also been described in Gram-positive bacteria. Certain strains of *Streptococcus salivarius, Streptococcus gordonii, Streptococcus oralis*, and *Streptococcus pneumoniae* have short, thin, rodlike fimbriae. The flexible rodlike fimbriae described for various *Streptococcus, Enterococcus,* and *Corynebacterium* species and for the primary oral colonizers, *Actinomyces naeslundii* and *Streptococcus parasanguis*, have been shown to be important for adhesion and biofilm formation. In pathogenic strains they are known to play key roles in the adhesion and invasion process.

Unlike the pili of Gram-negative bacteria, Gram-positive pili are formed through covalent attachment of the pilin subunits to each other and to the peptidoglycan cell wall. For the streptococcal species, this has been shown to occur through recognition of an LPXTG amino acid sequence motif (where X denotes any amino acid) by an enzyme called **sortase**, which is a transpeptidase that catalyzes covalent peptide bond formation between the pilin subunits as they form a tandem chain of repeating subunits and ultimately a cross-linking bridge between the pilus and the peptidoglycan. Similar sortases and sortase-recognition motifs have been found in secreted pilin proteins in other Gram-positive bacteria.

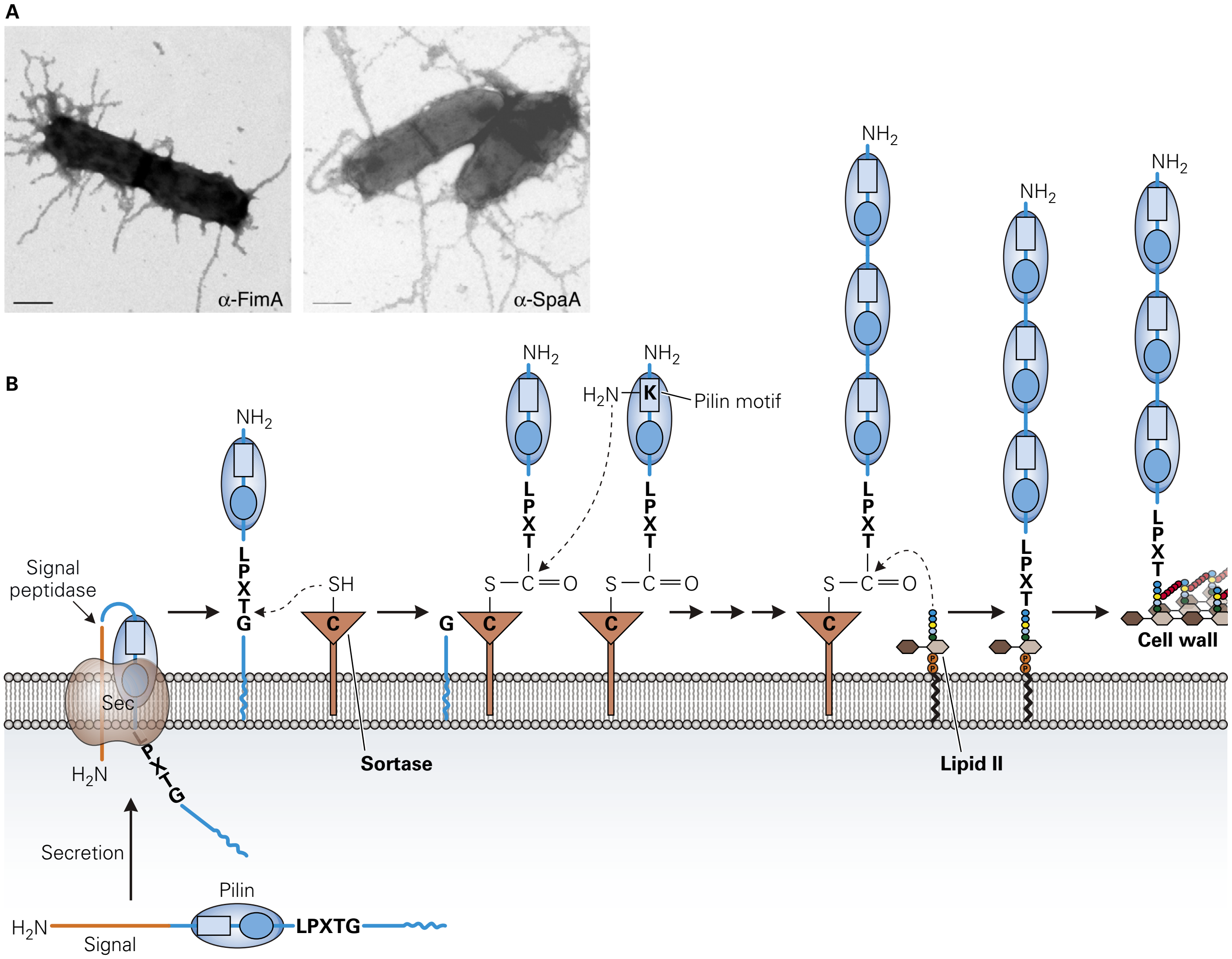


Figure 11-10. Model of pilus assembly in Gram-positive bacteria. (A) Electron micrographs of fimbriae from Gram-positive bacteria *Actinomyces naeslundii* (left panel) and *Corynebacterium diphtheriae* (right panel). Bacterial pili were detected by immunogold-labeling, first with the antibody against the major pilin proteins FimA or SpaA, respectively, followed by IgG conjugated with 12 nm gold particles. Reprinted from Ton-That H, Schneewind O. 2004. *Trends Microbiol* **12:**228–238, with permission. **(B)** Model of sortase transpeptidase-mediated pilus assembly in Gram-positive bacteria. Pilin subunits that contain the LPXTG motif (where X is any amino acid residue) are targeted to the cell membrane by the general Sec-dependent secretion system (SecYEG). Sortase cleaves in the motif between the T (threonine) and G (glycine) residues. A covalent thiol ester is transiently formed between the active site cysteine residue of the sortase and the carboxyl group of the threonine residue. Oligomerization occurs through attack of the thiol ester by the epsilon-amino group of the K (lysine) residue in the pilin subunit motif. After elongation of the pilus, the thiol ester of the sortase-coupled peptide is then attacked by an amino group side chain of the stem pentapeptide in a peptidoglycan precusor. The final precursor is a membrane-bound, covalently linked, covalently polymerized pilus. This precursor is then incorporated into the peptidoglycan.

A model for pili assembly in Gram-positive bacteria is shown in Figure 11-10. Specific sortases required for Gram-positive pilus assembly are often encoded by pathogenicity islands that also encode the pilin gene. Finally, it should be noted that Gram-positive bacteria produce at least one "housekeeping" sortase that covalently links many surface enzymes, adhesins, and virulence factors to the crosslinking bridges of the peptidoglycan. This covalent linkage anchors these proteins to the surface of the Gram-positive bacteria and prevents their escape into the medium.

Afimbrial Adhesins. As already mentioned, bacteria can use cell surface proteins for adherence that do not assemble themselves into pilus-like structures. These adhesins have been called **afimbrial adhesins** and probably mediate tighter binding of bacteria to the host cells, following initial binding via pili (see Figure 11-7F). Bacterial surface proteins are important components of the systems that allow bacteria to attach to and invade host cells (discussed in more depth later). The structure and function of only a small number of the afimbrial adhesins have been solved. At least some of them may recognize proteins such as integrins and cadherins on host cell surfaces rather than carbohydrates. Integrins are heterodimeric transmembrane proteins that maintain adhesive cell-to-cell interactions and interactions with the extracellular matrix, and they also transmit intracellular signals involved in receptor clustering and cellular invagination. Cadherins are calcium-dependent, transmembrane surface molecules that also play roles in host cell adhesion and signaling.

Some Gram-positive bacteria are covered by hairlike protrusions that resemble pili, and it was natural to assume that these structures might play the same adhesive role as the pili of Gram-negative bacteria. In at least one case, however, the assumption that fibrillar (pili-like) structures on a Gram-positive bacterium are adhesins now appears to be incorrect. The Gram-positive pathogen *S. pyogenes* is coated with a surface molecule that has a pilus-like α-helical structure consisting of many monomers of a protein, called **M protein**. At one time the pilus-like structures were thought to mediate adherence to fibronectin, a protein found on many host cell surfaces, and thereby help *S. pyogenes* adhere to the mucosa of the human throat when it causes strep throat or to tissue in wounds when it causes wound infections. Some M proteins can indeed bind fibrinogen to recruit blood platelets, which activates coagulation and thrombosis (blood clotting), but we now know that the M proteins have a very different function: inhibiting complement fixation and helping *S. pyogenes* avoid ingestion by phagocytes (discussed further later).

Many Gram-positive foodborne pathogens have nonfimbrial adhesins that bind to connective tissue and extracellular matrix constituents, which themselves serve as adherence substrates for eukaryotic cells. Various staphylococcal, streptococcal, and enterococcal species bind to fibronectin, collagen, fibrinogen, vitronectin, laminin, bone sialoprotein, elastin, or thrombospondin. Many of these extracellular matrix proteins are broadly distributed in the body (e.g., fibronectin is found in many different tissue types), while others are found only associated within defined tissue locations (e.g., laminin in basement membranes, sialoprotein in bone). This may contribute to the different tissue tropism of Gram-positive bacterial infections. The bottom line seems to be that Gram-positive bacteria, like Gram-negative bacteria, have pilus-type adhesins and afimbrial adhesins.

The Gram-positive bacterium *Listeria monocytogenes* uses two surface proteins called internalins A and B (InlA and InlB) to bind to host cells (Figure 11-11). *L. monocytogenes*, in susceptible individuals, can invade intestinal cells, infect mesenteric lymph nodes, the bloodstream, the spleen, and the liver, and ultimately cross the blood-placental and blood-brain barriers. InlA binds to E-cadherin, a transmembrane glycoprotein adhesion molecule found in host cells involved in the formation of intercellular tight junctions. InlB binds to three different receptors: gC1qR, the receptor for C1q (which is the first component of the complement cascade); Met, a hepatocyte growth factor tyrosine kinase receptor; and GAGs, glycosaminoglycans found on mammalian cell surfaces. In each case, binding of the bacterium to host cells triggers uptake of the bacterium by the host cells into vesicles that are not immediately targeted to lysosomes (discussed further later).

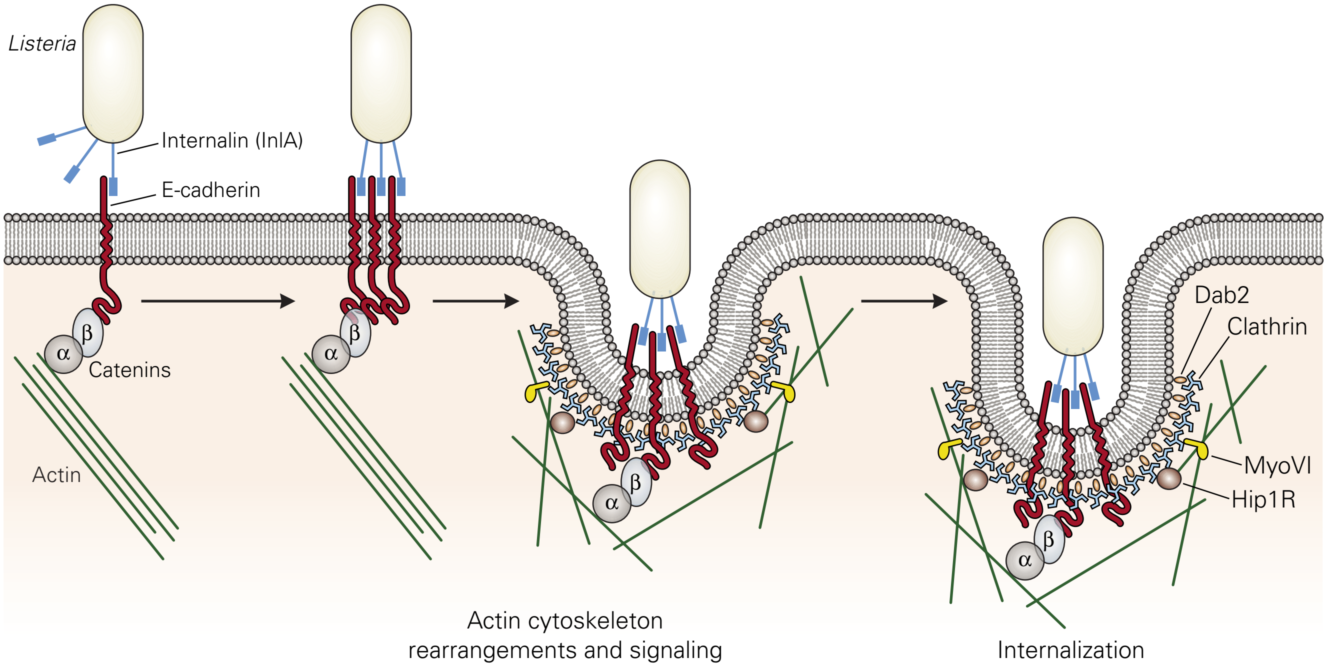
Attachment Triggers Host and Bacterial Signal Transduction. The original view of pili was that their only role was to form an attachment to human or animal cell surface molecules. It is now well established that attachment of adhesins also causes conformational changes in eukaryotic cell surface molecules that can trigger signaling cascades inside the eukaryotic cell, which can lead to cytoskeletal responses, including uptake of the bacteria, as well as altered expression of genes. The majority of integrin ligands contain the Arg-Gly-Asp (RGD) motif as the minimal protein sequence necessary for binding. Many afimbrial adhesins also contain this RGD motif. For example, *Yersinia* adheres to cells by recognizing α5β1-integrin through the bacterial protein invasin, which indicates that this invasin protein shares structural features (i.e., the RGD motif) with fibronectin, the natural host ligand for α5β1-integrin. Binding of invasin to α5β1-integrin triggers uptake of the bacterium into host cells. Intracellular bacteria have specialized adhesins that trigger bacterial uptake or invasion of the host cells (discussed in a later section).

Similarly, changes in conformation in the pilin tip proteins might cause a change in the shaft subunit proteins, which move along the length of the shaft to the bacterial surface, where they trigger changes in bacterial gene expression. A bacterium that has just attached itself to the surface of a mammalian cell exhibits altered expression of virulence genes, as if the act of binding triggered the activation or repression of virulence genes. We will explore this phenomenon more when we describe systems for direct delivery of bacterial virulence factors into eukaryotic cells in chapter 13 and for regulation of virulence factors in chapter 14.

Evading the Host Immune Response

The host immune response is an important parameter with which all incoming microbes must contend. Microbes are able to become pathogens because they have traits that enable them to avoid, neutralize, or counteract the innate or adaptive defenses, or both immune defenses, of the host. By

A. The InlA pathway


Listeria
Internalin (InlA)
E-cadherin
β
α
Catenins
Actin
Actin cytoskeleton
rearrangements and signaling
Dab2
Clathrin
MyoVI
Hip1R
Internalization

B. The InlB pathway

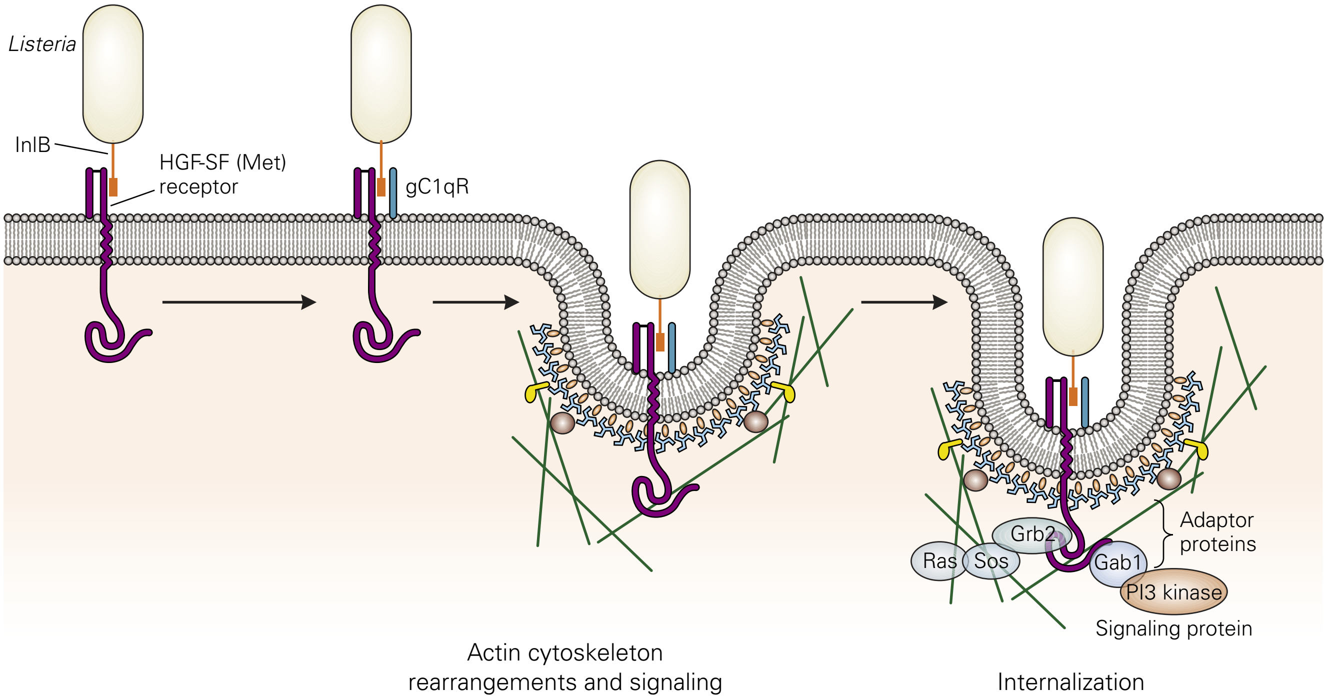

Listeria
InlB
HGF-SF (Met)
receptor
gC1qR
Grb2
Ras
Sos
Gab1
PI3 kinase
Adaptor
proteins
Signaling protein
Actin cytoskeleton
rearrangements and signaling
Internalization

Figure 11-11. ***Listeria monocytogenes*** **internalins and their host cell receptors. (A)** Internalin A (InlA) binds to E-cadherin, inducing receptor clustering. **(B)** Internalin B (InlB) binds to gC1qR and HGF-SF (Met) receptor. Binding of the internalins to their cognate host receptors triggers signal transduction pathways that lead to cytoskeletal rearrangements and endocytic uptake of the bacteria via the clathrin endocytosis machinery (Dab2, clathrin, Hip1R, and MyoVI). This complex serves as an initial nucleation point for actin cytoskeleton polymerization, which involves α-catenin and β-catenin in the case of E-cadherin or Ras, SoS, Grb2, Gab1, and PI_3 kinase in the case of gC1qR and Met receptors. Adapted from Pizarro-Cerdá J, Kühbacher A, Cossart P. 2012. *Cold Spring Harb Perspect Med* **pii:**a010009, with permission.

circumventing the host immune response, pathogens are able to take advantage of the nutrients and the growth-conducive environment of the host to survive, grow, proliferate, and disseminate. We will now take a look at some of the strategies pathogens have acquired or developed to survive in the face of hostile attack by the host immune system.

Avoiding Complement and Phagocytosis

Pili/Fimbriae. Bacteria growing in the body are constantly losing and reforming pili. Continuous production of new pili is necessitated in part by the fragility of pili, which are easily broken off through mechanical shearing. An equally important function of pilus replacement, at least for some bacteria, is that it gives them a way to evade the host immune response. Host antibodies that bind the tips of pili physically block the pili from binding to their host cell targets. Once the host begins to produce an antibody to a particular type of pilus, that pilus type is no longer useful to the bacteria. Replacing one type of pilus with another renders the host antibody response obsolete. Some bacteria, such as the bacteria that cause urinary tract infections (*E. coli*) and the bacteria that cause gonorrhea (*Neisseria gonorrhoeae*) can change pilin types often enough, by antigenic variation mechanisms (see chapter 7), to make it virtually impossible for the host to mount an effective antibody response that prevents colonization or allows for bacterial clearance.

Capsules. A **capsule** is a loose, relatively unstructured network of polymers that covers the surface of a bacterium (Figure 11-12). Most of the well-studied capsules are composed of EPS, but capsules composed of proteins or protein-carbohydrate mixtures have also been described (Table 11-2). The role of capsules in bacterial virulence is to protect the bacteria from the host's inflammatory response (complement activation and phagocyte-mediated killing). Recall that an essential first step in the alternative pathway is assembly of C3bBb (C3 convertase) on the bacterial surface (see Figure 3-14). Some capsules prevent formation of C3 convertase by failing to bind serum protein B. Other capsules have a higher affinity for serum protein H than for B. If C3b complexes with H rather than with B, then C3b is degraded by serum protein I. Capsules that are rich in sialic acid have a high affinity for protein H.

By preventing C3bBb formation on their surfaces, encapsulated bacteria gain some important advantages. The capsule itself is less likely to be opsonized, that is, phagocytes will not engulf the bacteria as efficiently. Some C3b may diffuse through the loose capsule network and bind to the bacterial surface under the capsule. C3 convertase may even form at this site, but the C3b molecules that attach to the bacterial surface under the capsular layer are physically blocked from making contact with phagocyte receptors by the thick intervening capsular network.

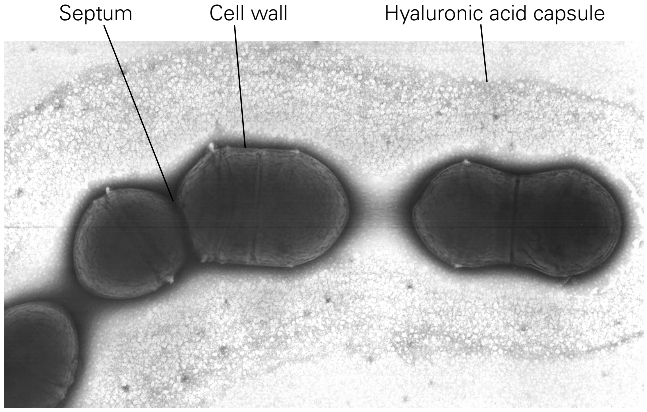


Figure 11-12. Capsule. Shown is a transmission electron micrograph of *Streptococcus pyogenes* (28,000x). The halo around the bacteria is the hyaluronic acid capsule. Courtesy of Vincent Fischetti.

Reduced C3bBb formation also means that less C5b will be produced, and hence the membrane attack complex (MAC) is less likely to form on the bacterial surface, an important consideration for Gram-negative bacteria. However, this is not always the case, and the MAC can still form on the surface of some encapsulated bacteria because proteins can diffuse through the loose network of the capsules. Thus, encapsulated bacteria do not automatically become serum resistant.

Mimicking the Host. An effective host response against an encapsulated bacterium is to produce antibodies that bind the capsule. Antibodies bound to the capsular surface not only provide sites for phagocyte binding so that bacteria can be ingested, but also support activation of complement by the classical pathway, thus increasing the amount of MAC formed. Vaccines consisting of capsular material have proven highly effective in preventing infections caused by encapsulated bacteria, such as *Haemophilus influenzae*, but some bacteria subvert this type of protective host response by having capsules that resemble host polysaccharides. Examples are capsules consisting of **hyaluronic acid** (an extracellular matrix polysaccharide) produced by *S. pyogenes* (see Figure 11-12), or **sialic acid** (a common component of host cell glycoproteins) found in some strains of *Neisseria meningitidis*. Because this type of modified capsule is not immunogenic, the host does not produce antibodies that opsonize the capsular surface.

One of the principal targets of complement on Gram-negative bacteria is LPS. LPS not only serves as a site for attachment of C3b, the signal for activation of the alternative pathway, but it also binds C5b and serves as a nucleation site for formation of the MAC (see Figure 3-13). Two types of LPS modification affect this interaction between LPS and complement components. First, attachment of sialic

Table 11-2. Composition of selected bacterial capsules

BACTERIUM	CAPSULE COMPOSITION
Gram-positive bacteria	
Bacillus anthracis	Poly-D-glutamic acid
Bacillus megaterium	Poly-D-glutamic acid, amino sugars, sugars, polypeptide
Streptococcus mutans	(Dextran) glucose
Streptococcus pneumoniae	Sugars, amino sugars, uronic acids
Streptocccus pyogenes	(Hyaluronic acid) *N*-acetylglucosamine and glucuronic acid
Gram-negative bacteria	
Acetobacter xylinum	(Cellulose) glucose
Escherichia coli	(Colonic acid) glucose, galactose, fucose, glucuronic acid
Pseudomonas aeruginosa	Mannuronic acid
Azotobacter vinelandii	Glucuronic acid
Agrobacterium tumefaciens	(Glucan) glucose

acid to LPS O antigen prevents formation of C3 convertase, similar to what happens with the sialic acid modified LOS of *N. gonorrhoeae*. Second, changes in the length of the LPS O-antigen side chains can prevent effective MAC formation, and hence bacterial killing by MAC does not occur. It is not entirely clear how O-antigen side chain length prevents MAC killing, since C5b and some MAC components still attach to the O-antigen. It is possible that the MAC forms too far away from the bacterial outer membrane to reach the bacterial membrane and exert a bactericidal effect. Bacteria that are not killed by the MAC are called **serum resistant**. An indication of the importance of this trait is that many of the Gram-negative bacteria that cause systemic infections are serum resistant (see Box 11-2).

Other Strategies to Avoid Complement and Phagocytosis. Bacteria have evolved strategies designed to prevent migration of phagocytes to the site of bacterial colonization. A bacterial enzyme that specifically degrades C5a, the chemoattractant for phagocytes, is produced by some Gram-positive bacteria and may be more widespread as a strategy for interfering with the signaling function of complement. Many bacteria produce toxic proteins (see chapter 12) that kill phagocytes, prevent their activation, inhibit their migration, block cytoskeletal changes needed for opsonization, or reduce the strength of the oxidative burst. Some bacteria can directly deliver these inhibitory toxins into host immune cells and block intracellular signaling pathways that modulate actin function or elicit cytokine and chemokine production (see chapter 12). Such toxins may protect the bacteria from phagocytes in the body.

Another strategy for evading complement activation is to have an LPS-type molecule that does not elicit the sort of strong host responses normally elicited by classical *E. coli* LPS. An example of a bacterial pathogen that has such an LPS is *H. pylori*. The O antigen of *H. pylori* LPS from certain strains contains carbohydrate moieties that are identical to human Lewis antigens x and y, both of which are found on the surface of gastric epithelial cells. This immune mimicry could help prevent the host from mounting an effective immune response. Despite this, *H. pylori* elicits an inflammatory

Box 11-2.

Bacterial Meningitis: An Example of the Power of Capsules

Bacterial meningitis is not a common disease, but it is a much-feared one, because it can kill an infected person within a few days. People who manage to survive a case of meningitis frequently have irreversible neurological damage, resulting in blindness, deafness, and learning deficiencies. A striking feature of meningitis is how rapidly it develops once the infection begins. This is due to the ability of the causative bacteria to divide rapidly in blood, a trait that is due to production of antiphagocytic capsules and to an ability to avoid complement-mediated killing. The most common causes of meningitis are *Neisseria meningitidis* (a Gram-negative bacterium that causes epidemic meningitis), *Streptococcus pneumoniae* (a Gram-positive bacterium that is also a common cause of bacterial pneumonia), and *Haemophilus influenzae* type b (a Gram-negative bacterium that was the most common cause of meningitis in children until a vaccine was developed).

What do these diverse types of bacteria have in common? All produce polysaccharide capsules, with the most dangerous strains being serum resistant. Once the bacteria gain access to the bloodstream, the capsule protects them from being killed by phagocytes. Although possession of a capsule does not automatically make a bacterium serum resistant, some strains of *N. meningitidis* become serum resistant by covalently attaching sialic acid residues to their LPS molecules. *H.*

Box 11-2.

Bacterial Meningitis: An Example of the Power of Capsules

influenzae type b strains can also become serum resistant by modification of LPS O-antigen side chains. *S. pneumoniae*, being Gram-positive, is naturally serum resistant since the membrane attack complex (MAC) does not form on most Gram-positive bacteria, due to the lack of an LPS-type molecule that can bind to the MAC. A strain of *E. coli* that causes meningitis in newborns (*E. coli* K1) also produces a capsule. This capsule is composed of sialic acid residues and thus does not bind C3b. In this case, the capsule has the same ultimate effect as serum resistance because it prevents MAC components from assembling in the first place.

The combination of a capsule and the ability of the bacteria to avoid being killed by complement renders all of the innate defense mechanisms of serum and blood ineffective, except transferrin-sequestration of iron. Unfortunately, *N. meningitidis* and *H. influenzae* can obtain iron from transferrin. The only defense that can check the growth of these bacteria in blood and cerebral spinal fluid is the antibody response. Antibodies to capsular polysaccharides opsonize the bacteria so that phagocytes can ingest them. Neutrophils and macrophages readily kill all of the bacteria mentioned earlier that are opsonized.

People who make antibodies against capsular antigens are fully protected from the disease. In fact, the reason *H. influenzae* type b is mainly a disease of children (aged 5 months to 5 years) is that most people who survive past age 5 have developed an antibody response against *H. influenzae*, probably as a result of transient nasopharyngeal colonization during childhood. Maternal antibodies protect newborn infants. There is now an effective vaccine against *H. influenzae* type b, which consists of capsular polysaccharide attached to a protein (a conjugate vaccine) to make it a better antigen (see chapter 17). This vaccine is now being used to protect children in the vulnerable younger age groups. There is also an older capsular polysaccharide vaccine against *S. pneumoniae* that is fairly effective. However, a newer conjugate vaccine against seven capsular antigens is highly effective in children.

Developing a vaccine against *N. meningitidis* proved a bit more challenging because the most common type of capsule in some parts of the world is type B, a capsule that (like the capsule of *E. coli* K1 strains) consists largely of sialic residues and thus does not evoke an antibody response. Vaccines against other types of *N. meningitidis* are available because these capsules are more immunogenic. There are now meningococcal vaccines licensed for use against three serogroups (B, C, and Y) that are commonly seen in the United States, as well as serogroups A and W found in other parts of the world.

MPSV4 is a polysaccharide-based vaccine targeting serogroups A, C, W, and Y that is used for children 2–10 years of age who are in high-risk groups, such as those who travel to countries where meningitis is endemic, those with terminal complement deficiencies, or those with a damaged spleen or whose spleen has been removed. MCV4 is a conjugate-based vaccine targeting either A, C, W, and Y or C, Y, and *H. influenzae* type b (Hib), depending on the manufacturer, that was licensed in 2005 and is recommended for all children 11 years or older and for adults who have never been vaccinated but fall into the high-risk groups listed earlier, who live in college dormitories or military barracks, or who might be exposed to meningococci at work (research or clinical). As a conjugate vaccine, immunity through MCV4 lasts longer than the MPSV4 vaccine, which needs boosters every 3–4 years. There is now also a recombinant meningococcal vaccine available against serogroup B.

During outbreaks of meningitis caused by *N. meningitidis*, as many as 80% of people in an exposed population may have their noses and throats colonized by *N. meningitidis*, but only a small percentage of the population colonized by the bacteria actually develop the disease. One possible explanation is that most adult members of a population may have some level of acquired immunity to *N. meningitidis* infection. A factor that clearly affects host susceptibility is the integrity of the mucosal membranes of the nose and throat, through which the bacteria usually gain access to the bloodstream. Outbreaks of *N. meningitidis* meningitis in Africa are almost always associated with the dry season, when dry conditions undermine the protective barriers of the mucosal membranes. Similarly, outbreaks in other countries appear to coincide with dry periods of the year. Acute viral respiratory infections such as influenza may also be a predisposing factor since such infections kill ciliated cells and undermine the integrity of the mucosal barrier. Interestingly, people with deficiencies in late complement components, who are unable to form the MAC, are also more prone to meningitis. Thus, although meningitis-causing strains are often somewhat serum resistant, the MAC may still exert some protective effect, at least in the case of the Gram-negative pathogens.

Why do people with meningitis die so quickly? The answer is that release of toxic cell wall components, such as LOS and peptidoglycan fragments, triggers a cascade of events leading to toxic shock and death. This type of bacterial toxicity was discussed in detail in chapter 3. Since the compounds that cause the symptoms of meningitis are released when bacteria lyse, administration of antibiotics, such as penicillins and cephalosporins that lyse bacteria, can actually temporarily worsen the condition of a patient, who has developed high numbers of bacteria in blood or cerebral spinal fluid. To counter this type of effect, which is most likely to occur in children because of the unusually high levels reached by bacteria infecting this age group, clinicians routinely administer corticosteroids along with antibiotics in an attempt to counteract the inflammatory effect of toxic compounds released from lysing bacteria.

response that in some individuals can result in gastritis and even gastric ulcers (Figure 11-13). If the host did respond by recognizing the Lewis antigen portions of the LPS molecule as foreign, the antibodies would cross-react with gastric mucosal cells. Such cross-reacting antibodies could contribute to inflammation by activating complement or stimulating the phagocytic cells to attack gastric epithelial cells. This may explain why patients with symptomatic disease often have antibodies that cross-react with antigens on gastric mucosal tissue.

Invasion and Uptake by Host Cells

One successful strategy used by a number of pathogens to avoid the host immune system is to hide from it by invading and living inside the host cell, where they are no longer exposed to complement or neutralizing antibodies. Pathogens that reside within host cells are referred to as intracellular pathogens. If the pathogen can avoid or survive phagolysosomal killing once inside the host cells, then they have the potential to gain access to a plethora of nutrients. Indeed, many obligate intracellular pathogens that have coexisted with their host for eons have evolved minimal genomes, where most nonessential genes used in other bacteria to survive in the external environment and genes used to synthesize nutrients that can be obtained readily from the host cell are deleted or rendered nonfunctional (so-called **pseudogenes**). *Chlamydia, Rickettsia, Treponema,* and *Coxiella* are examples of such obligate intracellular pathogens that can no longer reproduce outside of their hosts.

Many intracellular bacteria have evolved mechanisms for entering host cells, such as epithelial cells, that are not naturally phagocytic. They do this by attaching to the host cell surface and causing changes in the host cell cytoskeleton that result in their engulfment by the host cell. In actively phagocytic cells, cytoskeletal rearrangements involving po-

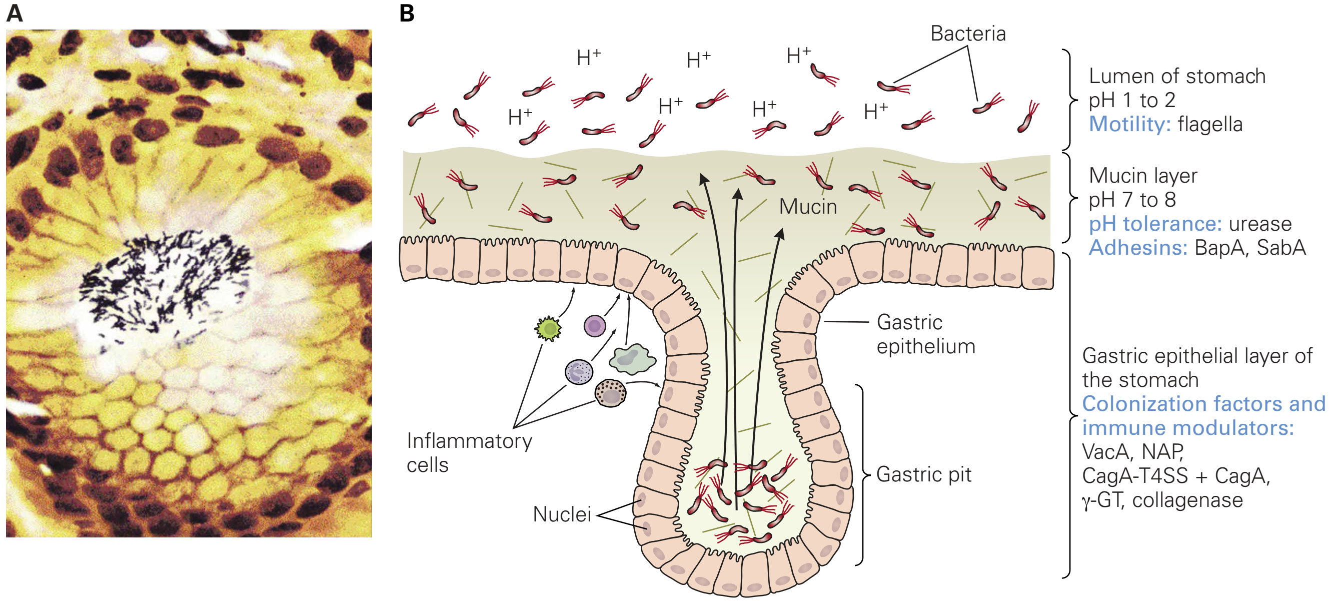


Figure 11-13. Proposed mechanism of gastric ulcer formation by *Helicobacter pylori*. (A) Shown is a photomicrograph of a biopsy specimen of a stomach pit from a patient with an active duodenal ulcer. The gastric pit is filled with *H. pylori*. The specimen was stained with Genta, a triple stain consisting of modified Steiner silver stain, hematoxylin and eosin, and Alcian blue, pH 2.5. Reprinted from Genta RM, Graham DY. 1996. *N Engl J Med* **335:**250, with permission. **(B)** Shown is an overview of *H. pylori* and host factors involved at the site of ulcer formation. *H. pylori* virulence factors thought to be involved at each stage are indicated at the right. Gastric ulcer formation, and later progression to gastric cancer, is the result of a complex set of interactions between virulence factors produced by the bacterium, host genetics and inflammatory responses, and environmental conditions. *H. pylori* virulence factors that provoke an inflammatory response that ultimately damages the mucosa include: urease (converts urea to ammonia, protecting bacteria from stomach acid), flagella (motility through mucin to reach epithelial layer), CagA (Cag-T4SS toxic effector protein), VacA (secreted autotransporter-type toxin), BabA (Lewis^{b} blood group antigen-binding adhesin), SabA (sialic acid-binding adhesin), NAP (neutrophil-activating protein) γ-GT (secreted γ-glutamyl transpeptidase), and a secreted collagenase. CagA- and VacA-producing s1m1 strain variants of *H. pylori* are associated with the severest forms of disease outcome. Salt concentrations in the stomach increase CagA production, and low iron levels increase T4SS-associated pili adherence to α5β1-integrin on epithelial cells and translocation of CagA into epithelial cells, where it modulates a number of cellular processes leading to proinflammatory responses, such as enhanced secretion of IL-8 and other cytokines/chemokines, mitogenesis, disruption of tight junctions, and other cellular effects. Host inflammatory responses that contribute to disease include IL-1β, IL-8, IL-10, IL-32, and TNFα. VacA is a T5SS autotransporter toxin that causes multiple toxic effects on epithelial cells, including vacuolation, induction of autophagy and apoptosis, and suppression of T cell responses, all of which contribute to persistence of infection.

lymerization and depolymerization of actin occur as an integral part of pseudopod formation. By causing similar actin rearrangements to occur in normally nonphagocytic cells, the bacteria are in effect forcing phagocytosis by eliciting formation of pseudopod-like structures that mediate engulfment. Bacterial surface proteins that provoke phagocytic engulfment of the bacterium by host cells are called **invasins**.

Some proteins that are essential for invasion of cultured cells by bacteria have now been identified. One of the first invasins discovered was that used by *Yersinia pseudotuberculosis* to gain entry into M cells of Peyer's patches and thereby transit into the underlying tissues. The *Yersinia* invasin (encoded by the *invA* gene) was found by randomly cloning *Yersinia* genes in *E. coli* and isolating clones that allowed the normally noninvasive *E. coli* strains to enter cultured mammalian cells. The mammalian receptors that are partners for invasin are members of the β_1 integrin family of cell adhesion molecules that are found on the apical surface of M cells in Peyer's patches. A number of cytoskeletal proteins are known to interact with the integrin receptors—including focal adhesion kinase (FAK), talin, α-actinin, and other kinases, as well as GTP-binding proteins—and invasin binding to the integrin receptors causes clustering and activation of the signaling, which in turn triggers actin rearrangements in the M cells and engulfment of the bacteria.

Eukaryotic cell biologists, especially those interested in the body's defense systems, view the process of invasion somewhat differently. While bacteriologists see the invasion process as one mediated by bacteria, with the purpose of entering and infecting a human cell, gastrointestinal cell biologists view the mucosal cells as sentinels monitoring bacterial invasion. Bacterial invasion of mucosal cells triggers these cells to release chemokines and cytokines that are known to be important for mobilizing phagocytes and other immune defenses. Although there are examples where attachment of pili or other bacterial surface proteins, such as specialized type 3 or type 4 secretion systems (discussed in chapter 13), can trigger signaling in the host cell, bacterial attachment alone is not always sufficient to start the signaling process that causes the body to mount an inflammatory response. However, any more intimate connection, especially disruption of normal actin polymerization processes and the presence of bacteria within mucosal cells, sets off the cytokine/chemokine alarm system that initiates the inflammatory response. And, in particular, as we learned in chapter 3, bacterial products such as LPS can trigger these inflammatory responses through activation of Toll-like receptors, without the involvement of uptake by phagocytes or bacterial invasion of host cells. In fact, LPS that has been released from lysed bacteria can also trigger inflammatory responses.

Surviving Phagocytosis

Bacteria that have been ingested by host cells are enclosed in a membrane vesicle called a **phagosome**, which then fuses with lysosomes to start an intensive attack on the bacterium called the **oxidative burst**. The vast majority of bacteria are killed by this very successful offensive strategy. There are bacteria, however, that manage to evade or survive and even multiply inside phagocytes. These are some of the most dangerous pathogens. The only effective host responses left against bacteria that can survive inside normal phagocytes are the activated macrophage response or the cytotoxic T cell response (see chapters 3 and 4). Bacterial pathogens have evolved a variety of mechanisms to survive inside phagocytes. A few of the best-known examples are described here.

Escape from the Phagosome. One strategy for avoiding being killed once inside a phagocyte is for the invading bacterium to escape from the phagosome before it merges with the lysosome. There are many advantages to be gained by escaping the vesicle and growing in the cytoplasm of host cells. These include an abundance of nutrients, protection from antibodies and complement, and partial protection from some antibiotics, as well as transport to other body sites if the infected cell is an immune cell or blood cell that travels. The only effective host defense against bacteria that do this appears to be the cytotoxic T cell ($CD8^+$) response or the natural killer (NK) cell response, which kills the host cells infected by the bacteria (via MHCI-antigen recognition), and thus exposes the bacteria to extracellular defenses like complement. Note that in this case activated macrophages would not necessarily be effective because the bacteria are in the cytoplasm.

Escape from the phagosome is mediated by a bacterial protein toxin that disrupts membranes either by degrading membrane lipids or by forming pores in the membrane. An example of a bacterium that induces its own uptake and then quickly escapes the phagosome is *L. monocytogenes* (Figure 11-14). One of the proteins responsible for the escape from the phagosome is a pore-forming hemolysin, called listeriolysin O (LLO), which is responsible for the zone of β-hemolysis seen around the bacterial colonies when *Listeria* is grown on blood agar plates. LLO has considerable amino acid sequence similarity with cholesterol-binding, pore-forming cytotoxins produced by other Gram-positive pathogens, such as *S. pyogenes* (streptolysin O, SLO), *S. pneumoniae* (pneumolysin, Ply), and *Clostridium perfringens* (perfringolysin O, PFO).

The importance of LLO for escape from the phagocytic vesicle was demonstrated by an experiment in which the LLO gene *hly* was expressed in the common soil Gram-positive bacterium *Bacillus subtilis*. When the resulting *B. subtilis* strain was incubated with a macrophage-like cell line, the bacteria were able to escape the vesicle and grow rapidly in the cytoplasm. The optimal pH for the membrane lytic activity of LLO is 5.5, similar to the acidic pH of the phagolysosome. LLO has very little cytolytic activity at pH 7. This is in contrast to other cytolysins, such as PFO, which does not have a low pH optimum. An advantage of LLO having this difference in pH optimum is that once *Listeria* has escaped from the phagosome, the lytic activity of LLO is reduced so that the host cell's membrane is not also lysed and the bacteria can replicate in the protective environment of the cytosol.

Prevention of Phagolysosomal Fusion. Many intracellular pathogens produce factors that allow them to prevent phagosome-lysosome fusion and promote sequestration of the bacteria in specialized vacuoles within the cytosol of the host. The mechanism by which phagosome-lysosome fusion is prevented is poorly understood. The most-studied examples are the obligate intracellular pathogens *Chlamydia* and *Coxiella* (Figure 11-15) and the facultative intracellular pathogens *Legionella, Salmonella, Mycobacterium,* and *Brucella* (Figure 11-16).

Obligate intracellular pathogens, such as *Chlamydia* and *Coxiella*, generally have two developmental life cycles that enable them to survive the external environment during transmission from host to host and the harsh environment of the phagocyte. In *Chlamydia* the extracellular infectious form of the bacterium that permits transmission from one host to the next is the small metabolically inactive elementary body (EB) (Figure 11-15A). The metabolically active form of the bacterium that replicates inside the host cell is the larger, noninfectious reticulate body (RB). After binding and uptake into cells, the EB is contained within a specialized vacuole, called the inclusion, where the EB converts into the replicative RB form. After two to three days the RBs convert back into EBs and are released from the cell through lysis, mediated by proteases and intracellular calcium, or by extrusion release of packaged EB-filled vesicles, mediated by Rho GTPase-regulated, actin-based cytoskeletal rearrangements.

Like *Chlamydia*, the metabolically inactive, small-cell variant (SCV) form of *Coxiella burnetii* is highly infectious because it can survive for long periods of time in the environment (Figure 11-15B). After the SCVs invade and form a *Coxiella*-containing vacuole (CCV), they convert into the metabolically active, large-cell variant (LCV) form that can replicate, but only after recruitment of the V-ATPase (proton pump) and acidification of the CCV. There is an initial delay in lysosomal fusion with the CCV, during which interaction with components of autophagy occurs, as well as recruitment of degradative cathepsin D, acid phosphatase, and various other proteins that protect the bacteria from phagolysosomal

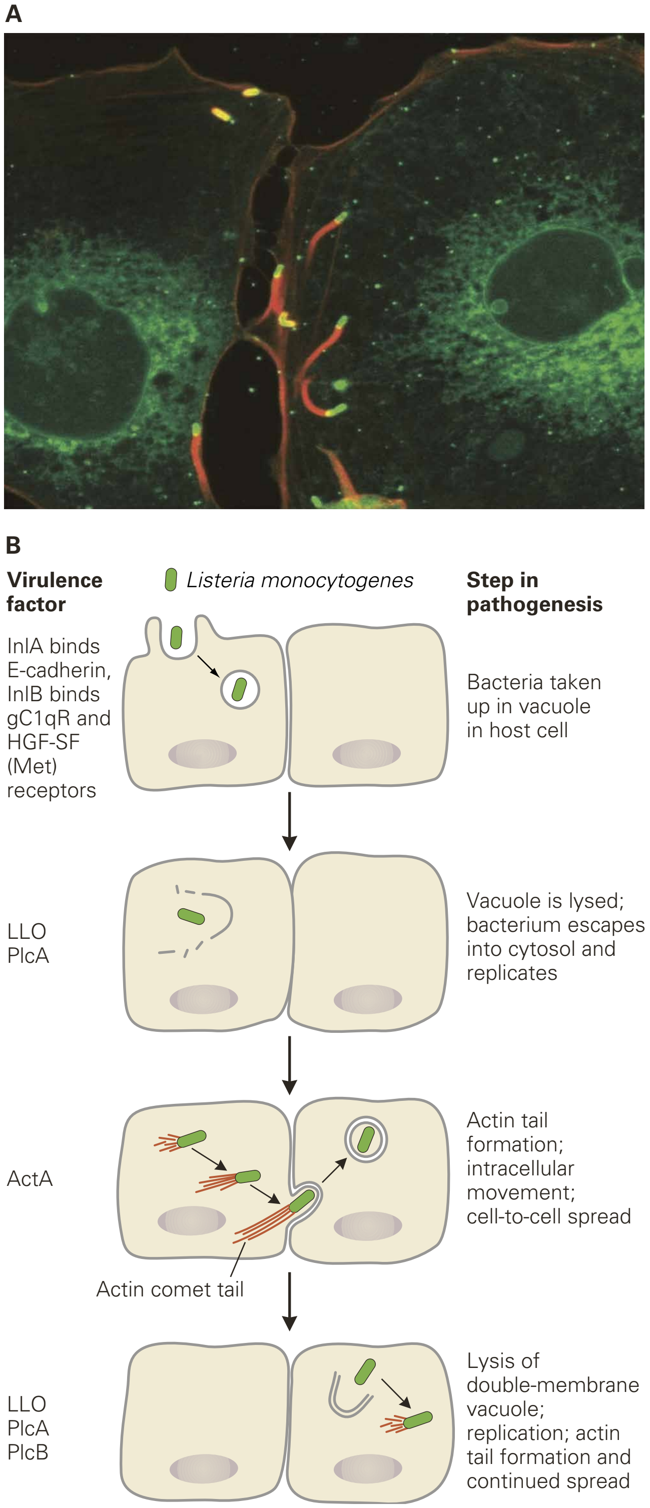

A
B
Virulence factor
Listeria monocytogenes
Step in pathogenesis
InlA binds E-cadherin, InlB binds gC1qR and HGF-SF (Met) receptors
Bacteria taken up in vacuole in host cell
LLO
PlcA
Vacuole is lysed; bacterium escapes into cytosol and replicates
ActA
Actin tail formation; intracellular movement; cell-to-cell spread
Actin comet tail
LLO
PlcA
PlcB
Lysis of double-membrane vacuole; replication; actin tail formation and continued spread

Figure 11-14. The intracellular life cycle of *Listeria monocytogenes*. (A) Shown is a fluorescence micrograph image of the intracellular actin-based motility (actin comet tails labeled red) and cell-to-cell spread of *L. monocytogenes* (green). Reprinted from Schuppler M, Loessner MJ. 2010. *Int J Inflam* **2010**:704321, with permission. **(B)** Shown is a schematic diagram of the steps in *Listeria* entry, escape from phagosome, actin-tail formation, and cell-to-cell spread. The initial internalization step involving InlA or InlB is detailed in Figure 11-11. Upon uptake, the bacteria escape from the phagolysosome via LLO and PlcA. In the cytosol the bacteria replicate and recruit actin to the ActA protein, which is displayed at one pole of the bacteria. ActA mediates actin polymerization to form the characteristic comet tails, which promote cell-to-cell spread to the adjoining host cell, where LLO, with the help of PlcA and PlcB, mediates escape from the double-membrane vacuole.

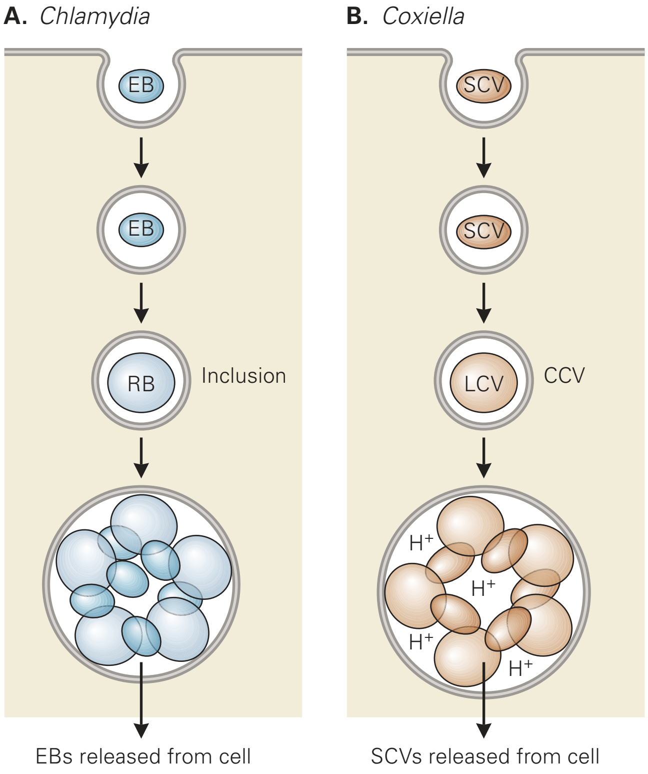


Figure 11-15. Obligate intracellular pathogens have two developmental forms that permit survival outside and inside the host. (A) The extracellular, infectious form of *Chlamydia* is the small, metabolically inactive elementary body (EB), while the metabolically active, replicative form of the bacterium is the larger, noninfectious reticulate body (RB). After binding and uptake into cells, the EB is contained within a specialized vacuole, called the inclusion, where the EB converts into the replicative RB form. After two to three days, the RBs convert back into EBs and are released from the cell. **(B)** Like *Chlamydia*, the metabolically inactive, small-cell variant (SCV) form of *C. burnetii* is highly infectious. After invasion and formation of the *Coxiella*-containing vacuole (CCV), SCVs convert into the metabolically active, large-cell variant (LCV) form that, after acidification of the CCV, can replicate before converting back into SCVs, which are then released from the cell four to seven days later.

oxidative burst conditions. LCVs replicate in an expanding CCV for four to seven days before converting back into SCVs, which are then released from the cell.

Unlike obligate intracellular pathogens, facultative pathogens can survive and replicate both outside the host in the external environment and within the host phagocyte. These pathogens have evolved a myriad of unique and elegant strategies for survival under each condition. The strategies used by *Legionella* are remarkably sophisticated in their ability to manipulate mammalian phagocytic cells (Figure 11-16A). This is evident from a comparative study of attachment and invasion and from what happens to the phagosomes after invasion has occurred. *Legionella* can enter macrophages even in the absence of opsonization by C3b or antibody. The macrophage invasin Mip appears to facilitate the uptake process. Cells are induced to undergo a coiling type of phagocytosis, resulting in a vacuole that is covered with ribosome-studded ER. Once the bacteria are internalized inside a vacuole, a complex developmental process begins. Not only do the vacuoles not acidify to the same extent as normal phagosomes, but they also leave the normal pathway that leads to phagolysosomal fusion. This seems to be due to a combination of pH homeostasis and elimination of lysosomal membrane proteins, such as LAMP-1 and LAMP-2, that are required for fusion of lysosomes with phagosomes. The resulting vacuole becomes an incubator for bacteria, and eventually they escape from the vacuole into the cytoplasm, where they replicate until the cytoplasm is depleted of nutrients. They then lyse the host cell and spread to other cells.

Salmonella Typhimurium (Figure 11-16B) triggers actin rearrangements that lead to the formation of ruffle-like structures in the host cell membrane. The ruffles grow into pseudopods that encircle and engulf the bacteria, taking them up into spacious vacuoles. In these special *Salmonella*-containing vacuoles, the bacteria replicate. *Salmonella* actively remodels this compartment, which is distinct from a phagosome, using an entourage of bacterial proteins that it delivers into the host cell, called effectors (more on this in chapter 13), to establish a protected, growth-conducive environment in which the bacteria can replicate, while evading the host immune response. The vacuoles contain some lysosome-associated membrane protein markers, such as LAMP-1 and LAMP-2, but phagolysosomal maturation is stalled and the vacuoles do not acquire other markers, such as the mannose 6-phosphate receptor and lysosomal hydrolytic enzymes.

Mycobacterium tuberculosis (Figure 11-16C) binds directly to macrophage surface protein CR3, the normal receptor for iC3b, or the CR4 receptor. Another component that is important for mycobacterial entry is cholesterol, which is relatively abundant in mammalian plasma membranes. Evidence supporting the importance of cholesterol for entry comes from experiments in which membranes depleted in cholesterol had reduced ability to take up mycobacteria. Once again, binding is followed by internalization of the bacteria into phagosomes, which do not fuse with lysosomes. *M. tuberculosis* uses multiple strategies for impeding phagocytic destruction. Bacterial recruitment of several host proteins to the phagosome surface prevents phagolysosomal fusion at the early endosome stage. One such protein is TACO (for tryptophan-aspartate-containing coat protein) that blocks lysosome fusion and prevents endocytic acidification by blocking recruitment of lysosomal hydrolases and the membrane protein (V-ATPase) that pumps protons into the endosome. TACO also binds the small GTPase Rab5, but not Rab7 or Rab5 proteins, and in doing so modulates downstream signaling. *M. tuberculosis* secretes a

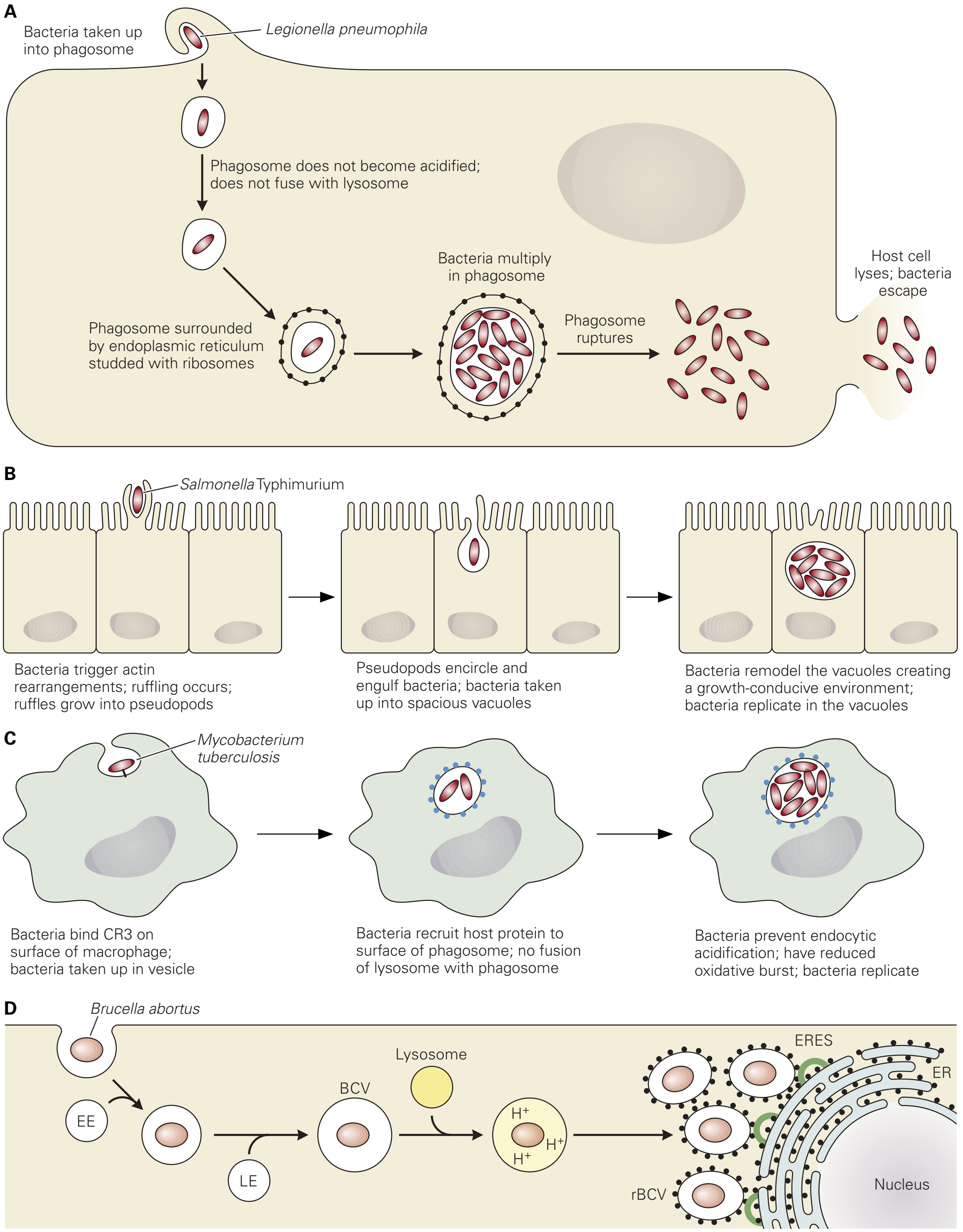

A
Bacteria taken up into phagosome
Legionella pneumophila
Phagosome does not become acidified; does not fuse with lysosome
Phagosome surrounded by endoplasmic reticulum studded with ribosomes
Bacteria multiply in phagosome
Phagosome ruptures
Host cell lyses; bacteria escape
B
Salmonella Typhimurium
Bacteria trigger actin rearrangements; ruffling occurs; ruffles grow into pseudopods
Pseudopods encircle and engulf bacteria; bacteria taken up into spacious vacuoles
Bacteria remodel the vacuoles creating a growth-conducive environment; bacteria replicate in the vacuoles
C
Mycobacterium tuberculosis
Bacteria bind CR3 on surface of macrophage; bacteria taken up in vesicle
Bacteria recruit host protein to surface of phagosome; no fusion of lysosome with phagosome
Bacteria prevent endocytic acidification; have reduced oxidative burst; bacteria replicate
D
Brucella abortus
EE
LE
BCV
Lysosome
H+
H+
H+
ERES
ER
rBCV
Nucleus

Figure 11-16. Various mechanisms employed by facultative intracellular pathogens to survive inside phagocytes. (A) *Legionella pneumophila* are taken up in a vacuole (phagosome) that does not become acidified and does not fuse with a lysosome; instead, the vacuole becomes surrounded by endoplasmic reticulum-derived membranes. The bacteria replicate in the vacuole, escape the vacuole, and multiply in the cytoplasm before lysing from the cell. **(B)** *Salmonella* Typhimurium is taken up into a phagosome in the macrophage. Phagosome-lysosome fusion is prevented. The bacteria replicate in the specialized *Salmonella*-containing vacuole. **(C)** *Mycobacterium tuberculosis* is taken up into a vacuole (phagosome). The bacterium prevents phagolysosome fusion by recruiting host proteins to the phagosome, while blocking recruitment of lysosomal hydrolases, so no acidification occurs. The bacteria replicate in the resulting vacuole. **(D)** After internalization, the *Brucella*-containing vacuole (BCV) traffics through the endocytic pathway from the early endosome (EE) to the late endosome (LE). Acidification of the BCV triggers trafficking to ER exit sites (ERES), where it forms a replicative BCV (rBCV). Egress from the host cell and spread to adjoining host cells, which is still poorly understood, occurs after about three days.

phosphatase, SapM, that hydrolyzes membrane phospholipid PI_3P and prevents transition to late endosomes. The bacterium also has multiple mechanisms for imparting resistance to oxidative burst and reducing production of IL-12, a cytokine that stimulates Th1 responses (see chapter 4).

Most *Brucella* bacteria are usually killed within phagolysosomes of macrophages, such that less than 10% of internalized bacteria survive to establish a replicative compartment within the cell. *B. abortus* traffics among three subcellular compartments during its intracellular life cycle (Figure 11-16D). After internalization, the *Brucella*-containing vacuole (BCV) traffics through the endocytic pathway, acquiring many components of early (Rab5, EEA1) and late (Rab7, LAMP-1) endosomes along the way. Acidification of the BCV after recruitment of V-ATPase triggers transport to and emergence with the ER at ER exit sites, where it forms a replicative BCV (rBCV). Egress from the cell, which is still poorly understood, occurs after about three days and appears to involve formation of an autophagosome-like vacuole (aBCV) that promotes spread to adjoining host cells. Another exit strategy is for the *Brucella* bacterium to spontaneously convert from smooth (with normal LPS) to rough mutants (with truncated LPS O antigen). Rough *Brucella* are cytotoxic to phagocytes and cause cell lysis.

Neutralization or Resistance to Phagolysosomal Components. One strategy for surviving phagocytosis is to acquire traits that reduce the effectiveness of the toxic compounds released into the phagolysosome after fusion occurs. Examples include bacterial cell membranes with altered structures, modified LPS, or capsules that provide resistance to killing by defensins or that are refractory to destruction by lysosomal proteases and lysozyme. Some bacteria are acid-tolerant and can survive for periods of time within the acidic phagolysosomal compartments. For example, *C. burnetii*, the causative agent of tick-borne Q fever, is an obligate intracellular pathogen that is remarkably resistant to the conditions found within the phagolysosome. Indeed, *C. burnetii* is able to not only reside, but also replicate, within specialized vacuoles that it helps form through modulation of the host machinery, whereby certain lysosomal components, such as cathepsin D and acid phosphatase, are still allowed to accumulate in the bacteria-containing phagosomes, while full lysosomal fusion is delayed (Figure 11-15B). *Coxiella* employs the acidic environment of these degradative, phagolysosomal-like compartments with pH ~5 (i.e., conditions where other bacteria cannot survive) to activate its metabolic activity and express an arsenal of proteins that protect the bacterium from phagocytic oxidative burst.

Resistance to Reactive Oxygen and Nitrogen Species. Many bacterial pathogens have evolved mechanisms to survive the harsh environment of phagolysosomes. Some of these mechanisms include production of enzymes, such as catalase and superoxide dismutase (Sod), that neutralize reactive forms of oxygen; cell surface polysaccharides that interact with and detoxify oxygen radicals; or cell surface proteins or glycolipids that reduce the strength of the oxidative burst. For instance, *M. tuberculosis* has a thick cell envelope that is comprised of an abundance of oxygen and nitrogen radical scavengers, such as lipoarabinomannan and phenolic glycolipids, which provide intrinsic resistance to oxidative damage. Table 11-3 summarizes a few known mechanisms used by pathogens to protect themselves from phagocytic killing by oxidative burst and other redox stresses.

For example, *S. enterica* serovar Typhimurium, which replicates within macrophages, has a number of strategies to deal with reactive oxygen and nitrogen species (Figure 11-17). *Salmonella* produces four Sods: two cytoplasmic Sods (SodA and SodB) and two periplasmic Sods (SodC1 and SodC2). SodA and SodB protect against DNA damage and inactivation of enzymes caused by superoxide in the cytoplasm of the bacteria. Both of the periplasmic Sods are expressed during infection, but only SodC1, which is encoded by a functional prophage, is important for virulence in a mouse model of infection. SodC1 enhances survival within the macrophage during infection because it remains tethered within the periplasm through noncovalent binding to the peptide portion of peptidoglycan and is inherently resistant to proteases, such that it can provide protection from superoxide. Interestingly, mechanisms that protect the bacterial outer membrane against host-derived antimicrobial peptides, which would result in release of the SodC2 from the periplasm, allow SodC2 to remain in the periplasm and provide additional protection from superoxide.

Many bacteria possess thiol-dependent antioxidant defense systems, called **peroxiredoxins (Prx)**, that catalyze the reduction of hydrogen peroxide, organic hydroperoxides, and peroxynitrite. Bacterial Prx systems are comprised of two components: AhpC, which is a peroxidase with two catalytic Cys residues, and AhpF, which is a flavin-containing disulfide reductase that has two subdomains that act in concert as an NAD(P)H-dependent thiol/disulfide recycling component (Figure 11-18). In some bacteria, the recycling of AhpC and other Prxs is mediated by disulfide coupling with thioredoxin reductase (TrxR), which in turn is regenerated by thioredoxin (Trx) or via reaction by glutaredoxin (Grx). In many pathogens, such as *Salmonella*, the alkyl hydroperoxide reductase system has a dedicated flavoprotein disulfide reductase, AhpF, which has an N-terminal domain containing two Cys residues with structural homology to Trx linked to a C-terminal domain with homology to TrxR.

As described in chapter 3, nitric oxide (NO) is a reactive form of nitrogen that is produced by a number of cells of the human antibacterial defense systems. The strategies described previously that reduce O_2^- levels in bacterial cells also protect against highly reactive nitrogen species, such as peroxynitrite ($ONOO^-$), which can form through reaction of

Table 11-3. Selective examples of mechanisms used by bacteria to protect against phagocytic oxidative burst

BACTERIAL EVASION SYSTEM	MECHANISM OF ACTION	PATHOGEN EXAMPLES
Bacterial peroxiredoxins (alkyl hydroperoxide reductases) Cys-based peroxidases coupled with their cognate flavoprotein reductases (AhpC-AhpF, AhpC-TrxR/Trx, AhpC-Grx systems)	Conversion of hydrogen peroxide (H_2O_2) to water Conversion of peroxynitrite ($ONOO^-$) to nitrate (NO_3^-)	*Escherichia coli, Enterococcus faecalis, Helicobacter pylori, Legionella pneumophila, Salmonella* species, *Mycobateria, Staphylococcus* species, *Streptococcus* species, *Treponema pallidum, Pseudomonas aeruginosa*, *Burkholderia* species
Flavohaemoglobins (Hmp) Single-domain globins (Cgb)	Depletion of S-nitroso-thiols through depletion of the NO precursor FAD- and NAD(P)H-dependent conversion of NO into nitrate (NO_3^-)	*E. coli, Neisseri meningitidis, Salmonella enterica, Campylobacter jejuni*
Catalase (Kat)	Scavenging of hydrogen peroxide (H_2O_2)	*Mycobacterium tuberculosis*, many other pathogens
NADH dehydrogenase (NuoG/NAD-1)	Suppression of oxidative burst and phagocytosis	*M. tuberculosis*
NADH-menaquinone oxidoreductase type 2 (Ndh-2)	Mutations in Ndh-2 increase NADH levels, which decreases adduct formation of NAD with prodrugs isoniazid (INH) and ethionamide (ETA), increasing antibiotic resistance	*M. tuberculosis*
NO reductase	Conversion of NO to nitrate (NO_3^-)	*E. coli, Salmonella* species, *P. aeruginosa, Neisseria* species, *Staphylococcus* species
Phagocyte NADPH-oxidase inhibitors – truncated LPS forms of certain bacteria block activation – relocation of NADPH-oxidase to the plasma membrane	Suppression or inactivation of NADPH-oxidase enzyme, targeting NADPH-oxidase to the host cell plasma membrane and not the pathogen-containing vesicles	*Coxiella burnetii* *H. pylori*
Superoxide dismutases (SodA, SodB, SodC1, SodC2)	Scavenging of superoxide O_2^-	*E. coli, Salmonella* species, many other pathogens

O_2^- with NO. Additional ways that some bacteria resist killing by NO include conversion of NO into NO^{3-}. In *E. coli*, resistance to NO is mediated by a **flavohemoglobin**, also called nitric oxide dioxygenase (NOD), which is normally part of the bacterial respiratory system. Flavohemoglobin uses NADPH, flavin adenine dinucleotide (FAD), and oxygen (O_2) to convert NO into NO^{3-}. Ironically, the NO_3^- that is generated serves as a substrate for bacterial nitrate reductases, which in turn can boost NO_3^--dependent bacterial respiration and growth of the pathogen, such as *E. coli, Salmonella, Pseudomonas, Neisseria,* and *Mycobacteria*.

Although many bacteria produce proteins that make them somewhat resistant to reactive forms of oxygen and nitrogen, this alone is usually insufficient to protect them from the full oxidative burst of activated phagocytes. The most successful intracellular pathogens that survive and replicate within host cells have developed combinations of the survival strategies discussed previously, which in total help evade phagolysosomal killing.

Cell-to-Cell Spread

A couple of unique bacteria that force their own ingestion by controlling host cell actin organization continue to interact with actin once they enter the cytoplasm of the host cell. Here, condensation of actin on one end of the bacteria propels them through the host cell cytoplasm and into adjacent cells. The study of the various interactions between invasive bacteria and host cell cytoskeleton is an exciting area of current research on virulence mechanisms. One of the most fascinating aspects of this type of motility within host cells is that to date there are only three bacterial species, all pathogens and all from different bacterial genera, that are able to use this **actin-based motility** to promote **cell-to-cell spread**. One is the Gram-positive, foodborne pathogen *Listeria* species (e.g., *L. monocytogenes*) (see Figure 11-14). The second is the Gram-negative causative agent of dysentery, *Shigella* species (e.g., *Shigella flexneri*). The third is the causative agent of Rocky Mountain spotted fever, *Rickettsia rickettsii* (although not all *Rickettsia* species form actin tails). The ability of these intracellular pathogens to spread within host cells and tissues very effectively enables them to evade the host's humoral immune response.

The process of actin-based motility is illustrated here for *L. monocytogenes* (Figure 11-14). *L. monocytogenes* induces its own uptake into nonphagocytic cells and then escapes from the phagosome into the cytoplasm via the pore-forming

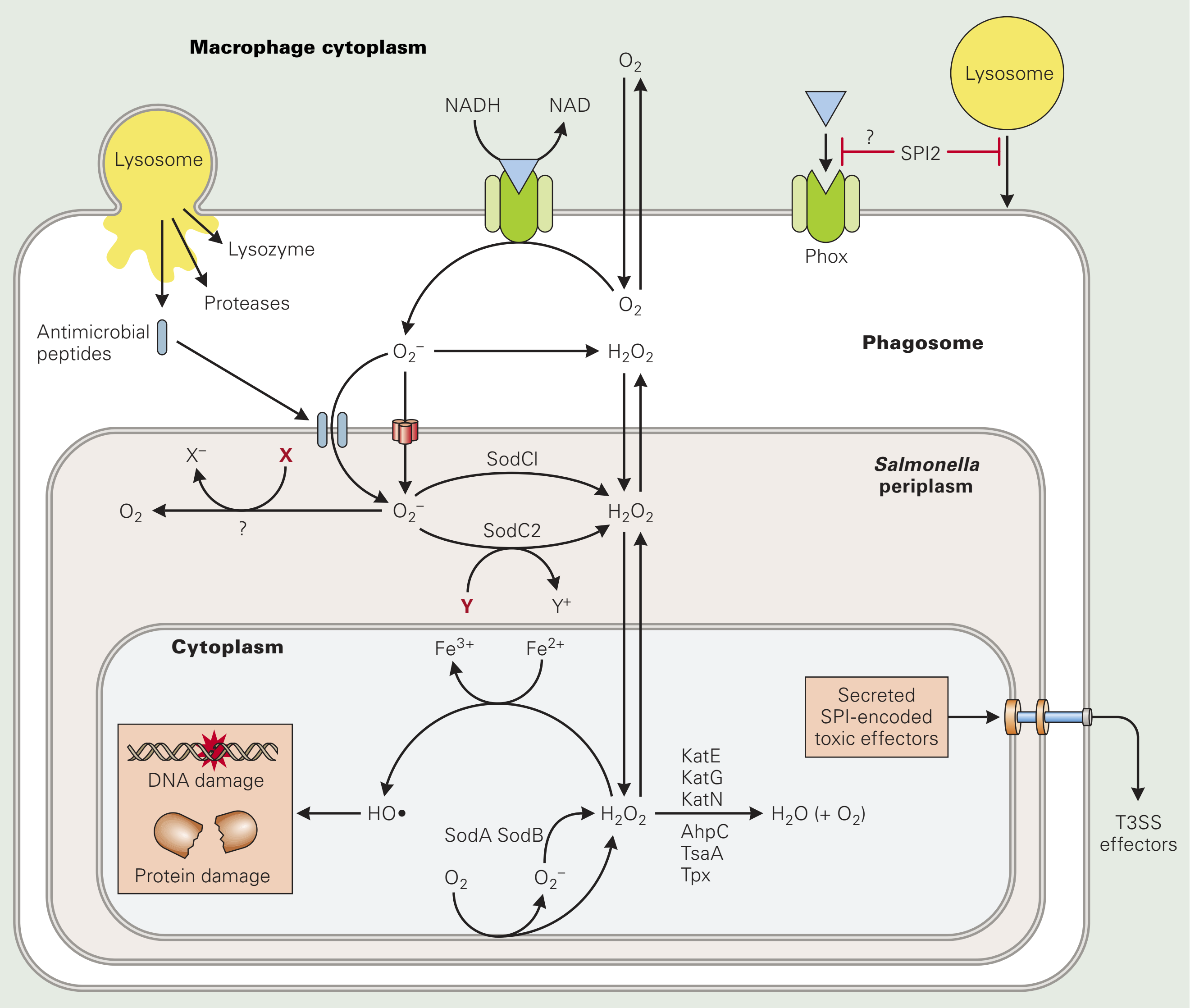

Macrophage cytoplasm
Lysosome
Lysozyme
Proteases
Antimicrobial peptides
NADH
NAD
O_2
O_2
Lysosome
?
SPI2
Phox
Phagosome
O_2^-
H_2O_2
Salmonella periplasm
X^-
X
O_2
?
SodCI
O_2^-
H_2O_2
SodC2
Y
Y^+
Cytoplasm
Fe^{3+}
Fe^{2+}
Secreted SPI-encoded toxic effectors
DNA damage
Protein damage
HO•
SodA SodB
H_2O_2
KatE
KatG
KatN
H_2O (+ O_2)
AhpC
TsaA
Tpx
O_2
O_2^-
T3SS effectors

Figure 11-17. Reactive oxygen species in the *Salmonella*-containing vacuole. The multisubunit NADPH-dependent phagocytic oxidase (Phox or NOX2) assembles at the vesicle membrane upon phagolysosomal fusion and reduces molecular oxygen (O_2) to generate superoxide (O_2^-). *Salmonella* survives within macrophages through multiple mechanisms, including producing three catalases (KatE, KatG, and KatN), three peroxidases (AhpC-AhpF, TsaA, and Tpx), and four superoxide dismutases (SodA, SodB, SodC1, and SodC2) that keep hydrogen peroxide (H_2O_2) levels low. This helps protect the cells against reactive oxygen species, such as O_2^-. SodC1—which, unlike SodC2, is tethered to the peptidoglycan in the periplasm—is critical for virulence because it protects as-yet-unknown periplasmic components (X, Y) from oxidative damage caused by O_2^-. *Salmonella* also produces toxic effector proteins that are directly secreted into the host cytosol and modulate phagolysosomal fusion and assembly of Phox.

toxin LLO as described earlier. Once in the cytoplasm, the bacteria are free to exploit the nutrient-rich environment to grow and replicate. The bacteria interact with host structural components to form actin tails.

The process of actin nucleation by the bacterium requires only one bacterial protein, a surface protein called **ActA** that assembles actin filaments by interacting with the host cytoskeletal proteins, profilin and Arp2/3. The bacterium polymerizes actin only at one end of the cell (where ActA is anchored) to form an actin tail (sometimes called an actin comet tail), such that only unidirectional movement occurs. Growth of the tail by actin polymerization at the end of the bacterium propels the bacteria through the cytoplasm, while the actin tail distal to the bacterium is subsequently depolymerized. *L. monocytogenes* can in this way move rapidly inside cells at rates of about 1.5 μm/s and then use this actin-based motility to propel themselves through the cytoplasm to penetrate other adjacent cells

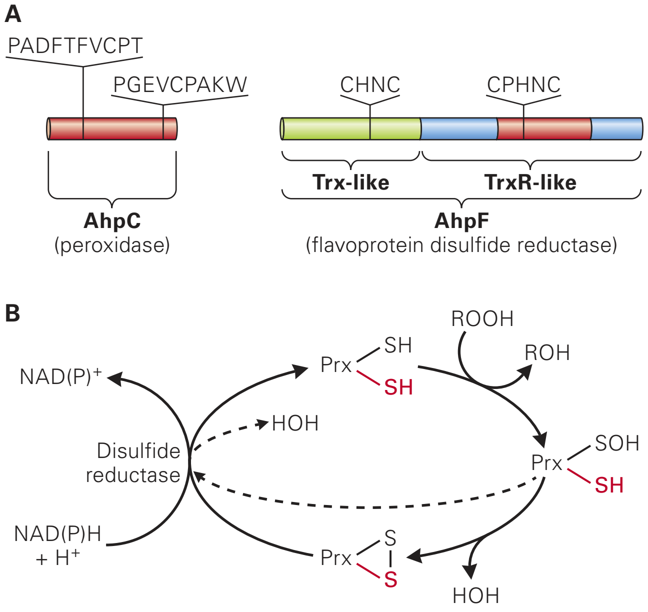


Figure 11-18. Peroxiredoxins as bacterial antioxidant defenses. (A) Shown is a schematic diagram of the AhpC peroxidase (left) and the flavoprotein disulfide reductase AhpF (right) proteins that are part of the bacterial alkyl hydroperoxide reductase system. The sequence motifs containing the catalytic Cys residues are indicated. The Trx-like N-terminal domain of AhpF is shown in green, while the TrxR-like C-terminal NADH-binding domain is shown in red and the FAD-binding domain in blue. **(B)** NAD(P)H-dependent thiol/disulfide recycling mediated by AphF. The side chain of one active site Cys cycles from free thiol (-SH) to sulfenic acid (-SOH) to disulfide (-S-S-) with the other reactive Cys side chain thiol (-SH).

(cell-to-cell spread). Once in the adjacent cell, the bacteria again use membrane-damaging toxins to escape from the double-membrane vacuole into the cytoplasm, where they once again continue to grow and spread from cell to cell.

Tissue Penetration and Dissemination

Many pathogenic bacteria produce factors that help them disseminate from the site of entry into the body to other body sites. A phagocytic attack on an invading bacterium will generate a region of dead cells. The material in the region is viscous **pus** consisting of DNA and proteins from dead phagocytes and other cells. This viscous pus material will trap some bacteria. Many bacteria, however, secrete **deoxyribonucleases (DNases)** that degrade DNA, thereby thinning the pus and making it easier for the pathogen to disseminate. Some bacteria produce **hyaluronidases** that degrade the charged polysaccharide **hyaluronic acid** in connective tissue, thereby also degrading the extracellular matrix and allowing spread along tissue planes. Other bacteria produce enzymes similar to **collagenases**, elastases, and other proteases that act as "meat tenderizers" to degrade connective tissue matrix proteins, thereby making it easier to spread outward from the area of entry and into areas that contain healthy tissues. The term "spreading factors" is sometimes used to refer to these enzymes collectively.

S. aureus can cause life-threatening infections, including "flesh-eating" skin infections (**necrotizing fasciitis**). Pathogenic strains produce a wide variety of degradative and proteolytic enzymes that aid in dissemination, such as **staphylokinase** (protease that dissolves blood clots), hyaluronidase, V8 protease (cleaves human IgG), and exfoliative toxins (cause scalded-skin syndrome, characterized by blistering and peeling of skin). The staphylokinase protease is a serine protease that acts as a plasminogen activator and cleaves plasminogen into plasmin, which then degrades fibrin clots and allows bacteria to escape from blood clots. Normally, the human body produces plasminogen activators for the same purpose, but with the aim of furthering wound healing.

Beyond Virulence Factors

Bacteria that appear to possess few or none of the virulence factors described thus far can nonetheless cause serious infections. Such bacteria are generally unable to cause infections in healthy people and preferentially infect people whose defenses are compromised in some way. Such bacteria are called **opportunists**. Despite their initial, seemingly harmless coexistence with us, opportunists can cause life-threatening infections. In fact, the most common causes of serious infections in hospitalized patients or cancer patients are the opportunistic pathogens. These include members of the body's normal microbiota, such as *Staphylococcus epidermidis*, *Enterococcus faecalis*, oral streptococci, clostridia, and *Bacteroides fragilis*, as well as common soil bacteria such as *B. cenocepacia* and *P. aeruginosa*. More details about these and other examples of Gram-positive and Gram-negative opportunistic pathogens will be discussed in chapters 18 and 19, respectively.

As some of these opportunistic pathogens are studied in greater detail, familiar virulence factors are often uncovered. For example, *B. fragilis,* a Gram-negative obligate anaerobe found in the colon, produces a capsule and has an altered LPS, and the Gram-positive *S. epidermidis* has cell-surface adhesins that bind extracellular matrix proteins and plastic surfaces. In some cases, such virulence factors play a somewhat different role than they do in the classical pathogens. For example, the capsule of *B. fragilis* seems to function less as an antiphagocytic mechanism than as a factor that elicits an inflammatory response. The surface adhesins of *S. epidermidis* allow it to bind tightly to medical implants rather than to mammalian cells.

Most of the bacteria that normally reside in the human body or in soil and water are not capable of acting as opportunistic pathogens, so the bacteria that do act as opportunists must have some special features that enable them to play this role. One feature is that they are constantly present in high numbers in the body or in the environment.

That is, they are on the spot to take advantage of any breach that occurs in the defenses of the human body. Another feature is that many of them are able to take advantage of locations in the body that are somewhat protected from the immune system. For example, *B. fragilis* and other opportunistic *Bacteroides* species tend to gravitate to damaged tissue. Damaged tissue is quite anoxic (lacking in oxygen) because it is cut off from the blood supply, so strict anaerobes, such as *Bacteroides* species, are quite happy to grow in these areas. *S. epidermidis* colonizes plastic implants, which are protected from phagocytes because phagocytes do not migrate as efficiently across the plastic surface as they do in tissue.

Still another feature that many opportunists have in common is their resistance to multiple antibiotics (see chapter 16). This not only gives them an advantage in the antibiotic-laden hospital environment, it also makes the infections they cause difficult to treat. The combination of impaired host defenses and a multidrug-resistant bacterium is a dangerous one, and one that is being seen more and more routinely in very sick hospital patients. This is the main reason why a bacterium like *S. epidermidis* or *E. faecalis* that is not very virulent can nonetheless kill the infected person. Another example of a very serious type of opportunistic infection that illustrates these principles is described in Box 11-1.

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Questions

1. Different parts of the host defense system work together to eliminate invading bacteria. Give some examples of how virulence factors could work together to make a pathogen better able to cause infection.
2. It was stated in the text that virulence factors might have arisen long before animals appeared on Earth. Of course, this is just speculation, but what arguments could be made to support this statement?
3. If the statement made in question 2 proves to be correct, what implications could it have for the number of bacteria able to cause human disease?
4. A bacterium has pili that allow it to attach to intestinal cells, after which it can invade the body. What type of vaccine could help prevent infections by such a pathogen?
5. In the case of an opportunist, what traits substitute for virulence factors? Could these traits be called virulence factors?
6. How could an actin structure such as the actin tail produced by *L. monocytogenes* cause movement of the bacteria?
7. A transposon insertion in *actA* stops tail formation by *L. monocytogenes*. Does this fact alone prove that ActA is an actin nucleator? What were some other possibilities scientists had to consider before concluding that ActA is the only bacterial protein that participates in the actin polymerization process? How could you determine if the actin polymerization factor was produced by the bacteria or is a host cell factor or protein?
8. How did scientists decide if LLO was important for escape from the phagosomal vacuole?
9. Infection with *L. monocytogenes* produces an antibody response, but this response is not very protective. Explain why this is so. Also, make an educated guess as to why adults exhibit only mild symptoms whereas intrauterine infections or infections of immunocompromised people can be serious.
10. One perplexing question that needs to be answered is why many people who are colonized with *H. pylori* never develop disease. What types of characteristics might explain why only a small percentage of people who are colonized by *H. pylori* ever develop ulcers?
11. How does the LPS of *H. pylori* differ in function from the LPS of most other pathogens?
12. Why has *P. aeruginosa* continued to be a major problem in hospitals?
13. Why might genetically eliminating adhesin genes have a much less drastic effect on LD_{50} values than eliminating genes for invasion or escape from the phagosome?
14. In the blurb at the beginning of the chapter, we state that bacteria with different disease MOs have different sets of virulence traits. From the examples given in the chapter, identify a few which exemplify this statement.
15. In 1990, a northeastern U.S. hospital noted an unusually high incidence of sepsis from *S. epidermidis* infections in their cardiac patients. The source of this mini-epidemic proved to be a surgeon. How might this episode have happened? (Hint: The surgeon had dermatitis. Also, in cardiac surgery, it is necessary to saw through the rib cage, creating many sharp bone fragments.)
16. Like *Pseudomonas*, *S. aureus* can form biofilms. What are the clinical implications if *S. aureus* growing in a biofilm is less susceptible not just to penicillin but also to vancomycin?
17. Bacteria able to grow intracellularly are often able to survive being phagocytosed by macrophages. What general strategies or properties might such a bacterium use to survive inside a host cell? What advantages might be provided by growing within a phagocytic cell?
18. Consider a bacterium that is ingested via contaminated water and locally infects the small intestine. What types of virulence factors would be most useful to this bacterium?
19. Describe at least four mechanisms that would allow a bacterium Y to compete with its host for iron.

Solving Problems in Bacterial Pathogenesis

1. A research group is studying bacterium X that binds to mucosal cells in the lung and invades. Wild-type X has an LD_{50} value of 10 bacteria when administered to mice by inhalation. Using transposon mutagenesis, the researchers have isolated two mutants of X that they call Xmut1 and Xmut2, both of which have LD_{50} values of 10^5 when inhaled by mice. However, in tissue culture cells Xmut1 can invade the cells just as well as wild-type X, while Xmut2 cannot. Provide a possible explanation for these results.

2. A group of researchers at the U.S. Department of Agriculture has isolated a new Gram-positive bacterium related to *Listeria ivanovii* from a recent outbreak of food poisoning due to contaminated cheese. They have determined that the wild-type bacterium can invade epithelial cells and spread from cell to cell. The researchers find that for intestinal epithelial cells, binding and uptake of *L. ivanovii* could be blocked by coincubation with galactose and lactose, as well as a mixture of crude gangliosides. Describe how they might go about experimentally identifying potential host cell surface receptors (glycolipids) involved in mediating the cellular uptake of the bacteria.

3. *Porphyromonas gingivalis* is associated with periodontal disease, including abscess formation and dental caries. Using conventional biochemical and genetic methods, a number of research groups have identified a variety of putative virulence factors thought to be involved in *P. gingivalis* pathogenicity. Among these are several fimbriae (e.g., FimA), a number of secreted proteases (e.g., PrtA cleaves IgA, PrtB cleaves IgG, PrtH cleaves C3, PrtP cleaves fibrinogen), a couple of hemin-binding outer membrane proteins (e.g., HemR), and a unique capsule that binds Factor H (CPS encoded by a large biosynthetic gene cluster).
   a. What are the possible roles of each of these seven bacterial factors (FimA, PrtA, PrtB, PrtH, PrtP, CPS, and HemR) in *P. gingivalis* pathogenesis?
   b. To verify that these factors are important for pathogenesis, a group of researchers made in-frame deletions of the genes encoding four of the putative virulence factors (FimA, PrtB, HemR, CPS), and tested the resulting mutants (Mu1-Mu4, respectively) in a mouse abscess infection model. Four groups of 30 mice each were injected subcutaneously with a 1:1 ratio of *P. gingivalis* wild type (WT) and one of the mutants. The surviving bacteria were then recovered daily for five days from the lesion sites of six mice in each group. The percentage of wild-type bacterial cells from the total number of bacterial cells recovered was then determined. The results are shown in the graph below. Interpret these results for each of the virulence factors tested in terms of their putative roles in pathogenesis using this infection model.

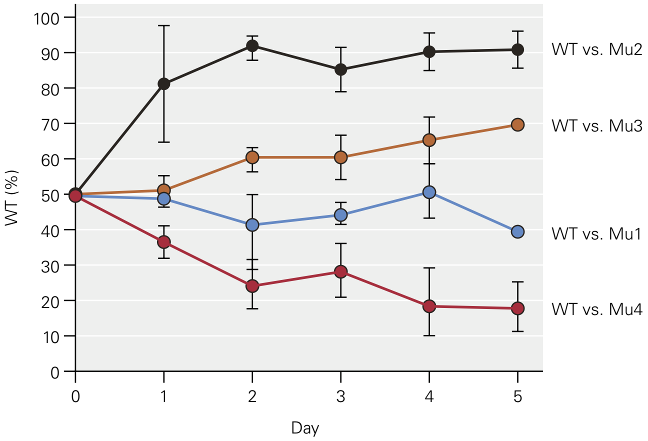

Figure 1. **Results.**

   c. Another group of researchers found that sera and inflamed gingival tissues of periodontal patients exhibited a positive antibody response to the cytoplasmic and surface-localized heat-shock protein (Hsp60) of *P. gingivalis*, which has significant sequence homology to human HSP60. Name possible implications of this finding with regard to immunity, pathogenesis, and vaccine development.

Special Global Perspective Problem: Integrating Concepts in Pathogenesis

Wild animals in zoos and rehabilitation facilities sometimes bite the animal handlers who care for them, a common occupational hazard. One of the animal caregivers at a zoo was bitten on the wrist while bottle-feeding a koala cub. Although the wound was cleansed and dressed, a tetanus-toxoid vaccine administered, and several oral antibiotics provided, erythema (redness), edema (swelling), and fever developed over the next two days, and additional antibiotics were given. Despite this, on the third day the symptoms worsened and the patient was admitted to the hospital, where surgical debridement of the wound was performed and further intravenous therapy administered. After six days the patient recovered and was discharged. Laboratory analysis of the specimens removed during surgery revealed small nonmotile Gram-negative rods; however, routine laboratory diagnostic phenotypic and biochemical methods failed to identify the organism. Subsequent 16S rRNA gene sequencing revealed that this bacterium was phylogenetically related to members of the Pasteurellaceae family, as shown in the dendrogram in Figure 1. The related *Lonepinella* strains (denoted with ACM numbers) were isolates from healthy koala bear feces, whereas the related *Actinobacillus* and *Pasteurella* strains were clinical isolates from diseased animals. The distinguishing features of *Lonepinella* are their rough colony morphology and their ability to degrade tannins found in *Eucalyptus* foliage, while the distinguishing features of *Pasteurella* are their smooth mucoid colony morphology and antiphagocytic properties.

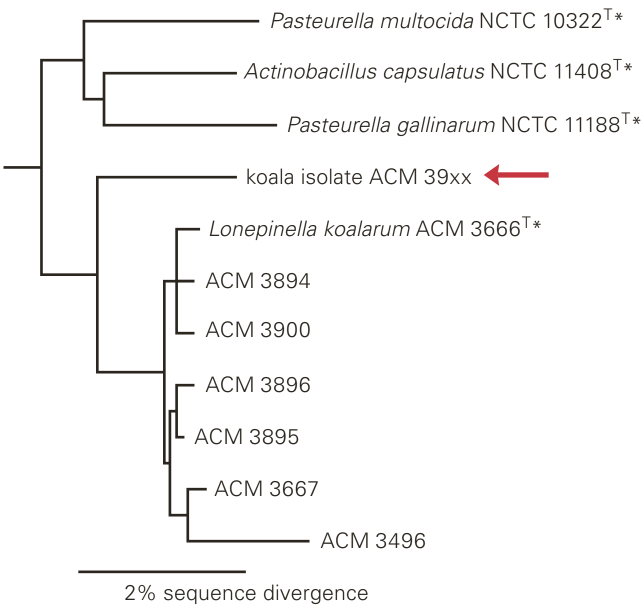
Pasteurella multocida NCTC 10322^{T}*
Actinobacillus capsulatus NCTC 11408^{T}*
Pasteurella gallinarum NCTC 11188^{T}*
koala isolate ACM 39xx
Lonepinella koalarum ACM 3666^{T}*
ACM 3894
ACM 3900
ACM 3896
ACM 3895
ACM 3667
ACM 3496
2% sequence divergence

Figure 1. Dendrogram of 16S rDNA-based phylogenetic relationships of the koala-bite isolate (arrow) to members of the *Pasteurellaceae* family. The phylogenetic tree includes strains of *Pasteurellaceae* isolated from other koalas (labeled ACM#) and well-characterized type strains (labeled T). Several of these bacteria have been genome sequenced (labeled with *).

1. You think that the unknown clinical isolate from the koala caregiver is a new virulent species of *Lonepinella* rather than *Pasteurella* or *Actinobacillus*, and you tentatively name it *Lonepinella koalababi* (Lkb) strain ACM 39xx. You also think that it may have picked up virulence traits from the *Pasteurella* or *Actinobacillus* strains through horizontal gene transfer (HGT). Without performing whole genome sequencing, provide three definitive experiments that you could perform to verify your hypothesis. Be sure to provide your rationale and expected outcomes.

2. You suspect that the new isolate Lkb has virulence properties that allow it to survive in the host to cause disease. You decide to conduct comparative genome sequence analysis of the new koala isolate Lkb strain ACM 39xx with that of the already-sequenced avirulent *L. koalarum* (Lk) strain ACM 3666, but in order to do so, you first need to sequence the Lkb genome.
   a. What sequencing technology would you choose to perform this? Be sure to provide your rationale for choice in terms of cost, efficiency, and outcome.
   b. Provide an overview of the experimental steps you would take to sequence the Lkb genome and identify genes that are unique to Lkb versus Lk.

3. You would like to identify which genes might encode virulence factors that enabled the new koala isolate to survive in the host and cause disease. As a first step toward accomplishing your goals, you decide to identify genes that are expressed only during infection in a mouse model of infection using Illumina RNA-Seq technology. From the transcriptome analysis shown in Figure 2, a single region of the genome showed differential expression in the animal.
   a. You hypothesize that this differentially expressed region is a pathogenicity island that is a transposon. Based on sequence analysis and the information given above, provide five pieces of evidence that would definitively support your hypothesis.
   b. Describe how you would verify that this region is indeed responsible for the observed virulence of Lkb.
   c. Provide an explanation for why the mRNA transcripts obtained for the *lkb03* gene do not cover the entire length of the predicted protein ORF.
   d. Based on sequence analysis of the operons under control of P1 through P6, the transcriptome analysis in Figure 2, and comparison of the gene sequences with DNA databases, you predict the following:
   - *lkb02* encodes a hyaluronic acid-binding adhesin.
   - *lkb03* encodes an anti-phagocytic outer-membrane protein with a protease-like C-terminal domain.
   - *lkb04* through *lkb06* are capsule biosynthetic genes.
   - *lkb07* encodes an iron-acquisition protein with a siderophore-binding domain at its C-terminus.
   - *lkb01* encodes a cryptic transposase, while *lkb08* encodes an active transposase.

   For each of the above set of genes, describe how you could experimentally confirm their predicted function.

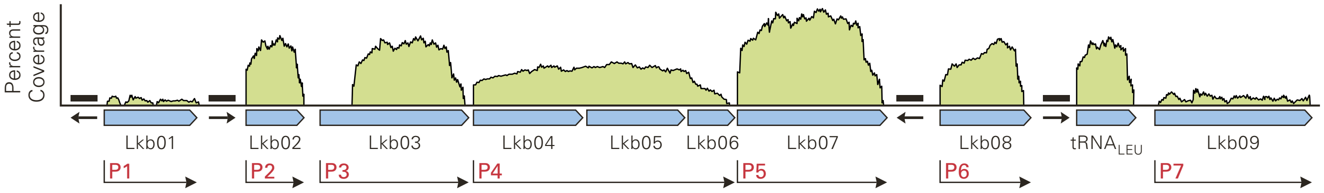

Figure 2. RNA transcript coverage for a region of the genome with differential expression *in vivo* versus *in vitro*. Predicted ORFs, labeled as Lk01 through Lk09, are indicated with directionality, predicted start sites with boxes, and promoters, labeled as P1–P7, with arrows. The tRNA for Leu is indicated. Dark arrows indicate repeat sequences.

12

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CHAPTER 12

Toxins and Other Toxic Virulence Factors

In the introductions to previous chapters we compared bacterial pathogens evading host defenses to criminals evading police detection. Continuing this analogy, criminals use a wide variety of tools to commit their nefarious deeds, ranging from crowbars and lockpicks to weapons like guns and knives. In this chapter, we consider the tools of choice in the microbial world. As different pathogens are faced with different challenges in breaching host defenses and maintaining a covert presence, they have evolved insidious and ingenious approaches to protect themselves, to countermand our alarm systems, and to create a comfortable, protected environment for themselves, typically at our expense.

Bacterial Toxins

Transparent Mechanisms, Exciting Applications, Mysterious Purposes

On the surface, diseases caused by bacterial toxins would seem to be the simplest and easiest to explain. The bacterium produces a substance (the toxin) that is toxic to human cells; the toxin causes the symptoms of disease; and the benefit of toxin action to the bacterium is clear. Oddly enough, this type of straightforward explanation has been surprisingly difficult to support in terms of the benefit to the pathogen. Toxins were the first virulence factors to be identified and studied in detail. In fact, scientists who studied toxins were the first ones to propose the concept of virulence factor—a single molecule that could cause disease symptoms. For a long time, toxins were the only virulence factors that could be clearly isolated and defined as having a role in pathogenicity. Since those early days, a vast number of bacterial toxins have been identified and studied, and our understanding of their biogenesis, regulation, structures, and functions in pathogenesis has advanced considerably. A number of toxin-based vaccines, generated through physical inactivation or protein engineering, are now available and have proven remarkably effective in protecting against toxin-mediated disease symptoms during infection. For example, the common childhood DTaP vaccine is a combination of the corresponding **toxoids** (inactivated, nontoxic versions) of diphtheria, tetanus, and pertussis toxins that is highly effective in protecting against diphtheria, lockjaw, and whooping cough, respectively (more on toxoid vaccines in chapter 17).

In terms of understanding their interactions with and toxic effects on the host, modern molecular biological, biochemical, and analytical technologies have made it increasingly easier to determine the cellular targets of toxins and to reveal the mechanisms by which toxins bind to cellular receptors, enter and traffic within the cell, penetrate or traverse membranes to gain access to their intracellular targets, and recognize and/or modify their intracellular targets to exert their toxic effects. Because toxins are often released or excreted into the extracellular fluid, most of them have been relatively easy to isolate, purify, and characterize. The crystal structures of many toxins, as well as toxins in complex with their cellular targets, have been solved. It is now clear that there is a staggering diversity of toxin structure, properties, and modes of action. Different toxins often have very distinct modes of action with clearly defined targets in mammalian cells; thus, it is not surprising that toxins have proven to be useful reagents for scientists studying cell biology and are increasingly being used as tools for probing signaling pathways and metabolic processes. Researchers are finding ever more exciting new ways to use toxins for beneficial purposes in medicine (e.g., to treat neurological conditions with botulinum neurotoxin) and agriculture (e.g., to control pests with insecticidal toxins).

Despite all these advances, we are still only at the precipice of understanding how and why bacteria acquire toxin genes and benefit from producing the toxins. Given the widespread production of toxins by bacteria and the number of toxins that are associated with human, animal, and plant diseases, it would seem to be a foregone conclusion that toxins are important for the survival and propagation of the bacterium that produces them. Importantly, there are an increasing number of cases in which this has been shown to be true. For example, toxins that kill or impair the action of neutrophils and macrophages can protect bacteria from the phagocytic cells that might clear them from the body. Toxins that kill human cells can release iron stores or carbon sources that the bacteria need to survive and multiply. Toxins can also act in more subtle ways, by dampening the immune response through modulation of the signaling pathways that regulate cytokine production, adhesion, proliferation, or migration. This **immunomodulation** enables the bacteria to survive in the host by evading the host immune system and to colonize and disseminate.

However, there are many cases in which it has been difficult to discern the benefits for the bacteria to produce the toxins. Toxins often have a clear function in causing disease symptoms in humans, but sometimes the role of a toxin in the propagation and evolution of the bacteria is not so obvious. A classic example of this is **botulinum neurotoxin (BoNT)**, the protein toxin produced by the Gram-positive bacterium *Clostridium botulinum*. BoNT attacks neurons in humans and animals and is responsible for the paralytic symptoms of the disease botulism, which can lead to death due to the inability to breathe. BoNT has impacted humans, because it is produced when the bacteria are growing in food. Nearly all cases of botulism result from foodborne disease caused by improper canning practices or from ingestion of clostridial spores present in raw foods, such as bee honey, unpasteurized organic juices, or inadequately washed vegetables. Thus, humans who ingest food containing the toxin can die of the effects of toxin that enters their nerves and causes paralysis. However, in adult humans most of the spores do not germinate immediately, and for those that do, the bacteria are unable to compete with the resident microbiota to colonize the intestinal tract, and so are eliminated rapidly from the body. By the time BoNT is produced and released so it can exert its effects, the bacteria are already on the way out of the body and gain no benefit from producing the toxin. In the case of foodborne disease from canned foods, the bacteria are long gone by the time of ingestion.

Why, then, does *C. botulinum* produce BoNT? *C. botulinum* normally survives as spores in the upper aerobic layers of the soil or as vegetative cells in lower anaerobic layers of the soil, in ponds, or in noncirculating water. In this environment, *C. botulinum* encounters fish and small mammals that may serve as nutrient sources when killed by a strong toxin. Other animals that eat these dead animals killed by BoNT may die themselves as collateral damage, one step removed from the environmental niche of this bacterium. Identifying the environmental niches where toxins foster the fitness of the bacteria that produce them remains an intriguing, and sometimes unsolved, mystery of modern pathogenesis research.

The plot thickens when one considers that in many toxin-producing bacteria, the toxin genes are not normal components of the bacterial genome. Comparison of the genomes of the sequenced toxin-producing bacteria with their nontoxigenic counterparts has revealed the abundance of toxin genes associated with pathogenicity islands located on extra-chromosomal or integrated plasmids, phage, or other mobile elements within chromosomes (Table 12-1). This strongly suggests that horizontal gene transfer (HGT) is a major driving force in the evolution of toxins and toxin-producing pathogens (see chapter 7). For example, when the toxin genes are carried on lysogenic bacteriophages, which integrate into the bacterial chromosome, only a bacterium that has acquired one of these phages produces the toxin (Figure 12-1). Non-toxin producing strains of *Corynebacterium* are often found as part of the normal microbiota of the throat. However, the *toxA* gene encoding **diphtheria toxin (DT)** is located on a corynebacteriophage, which is often harbored by *Corynebacterium diphtheriae* as well as other related *Corynebacterium* species. Exposure to these phage-infected bacteria can lead to rapid spread of the *toxA*-encoding corynebacteriophage among the resident corynebacteria. Likewise, there are many BoNT variants that are encoded by related *bot* genes found on phage/prophage, plasmids, and chromosomes of *C. botulinum* and other related *Clostridium* species. This suggests that widespread genetic exchange of *bot* genes among clostridia in the environment occurs through multiple HGT mechanisms.

Table 12-1. Properties of Selected Bacterial Toxins

TOXIN NAME	BIOLOGICAL ACTIVITY	GENE NAME	GENE LOCATION	BACTERIA THAT PRODUCE THE TOXINS
Single-peptide-chain AB-type toxins				
Clostridial neurotoxins	Zinc-dependent metalloprotease			
BoNT/A1		*botA1*	Chromosome	*Clostridium botulinum*
BoNT/A2		*botA2*	Prophage	*C. botulinum*
BoNT/B		*botB*	Plasmid	*C. botulinum*
BoNT/C1		*botC1*	Phage	*C. botulinum*
BoNT/D		*botD*	Phage	*C. botulinum*
BoNT/Dsa		*botC/D*	Phage	*C. botulinum*
BoNT/E		*botE*	Plasmid, phage	*C. botulinum, Clostridium butyricum*
BoNT/F		*botF*	Chromosome	*C. botulinum, Clostridium baratii*
BoNT/G		*botG*	Plasmid	*C. botulinum, Clostridium argentinense*
BoNT/FA		*botG/A*	Chromosome	*C. botulinum*
TeNT		*tet*	Plasmid	*Clostridium tetani*
Dermonecrotic toxins	G-protein-modifying deamidase and/or transaminase			
PMT		*toxA*	Prophage	*Pasteurella multocida*
DNT		*dnt*	Chromosome	*Bordetella bronchiseptica, Bordetella parapertussis, Bordetella pertussis*
Cytotoxic necrotizing factors	G-protein-modifying deamidase and/or transaminase			
CNF1		*cnf1*	Chromosome	*Escherichia coli*
CNF2		*cnf2*	Plasmid	*E. coli*
CNF3		*cnf3*	Chromosome	*E. coli*
CNFY		*cnfY*	Chromosome	*Yersinia enterocolitica, Yersinia pseudotuberculosis*
Diphtheria toxin (DT)	Elongation Factor 2-modifying ADP-ribosyltransferase	*Tox*	Corynebacteriophages	*Corynebacterium diphtheriae, Corynebacterium ulcerans, Corynebacterium pseudotuberculosis*
Pseudomonas exotoxin A (ExoA)		*exoA*	Chromosome	*Pseudomonas aeruginosa*
Shiga toxins	Protein synthesis inhibiting RNA *N*-glycosidase		Phages, prophages	
Shiga toxin (STx)		*stx*		*Shigella dysenteriae,*
Shiga-like toxin (SLT)		*stx1, stx2, stx1c, stx2c*		*Shigella sonnei, E. coli*
Clostridial toxins				
TcdA	G-protein-modifying UDP-glucosyltransferase	*tcdA*	Chromosome	*Clostridium difficile*
TcdB		*tcdB*		*C. difficile*
TcsH		*tcsH*		*Clostridium sordellii*
TcsL		*tcsL*		*C. sordellii*
Tcnα		*tcnA*		*Clostridium novyi*
Adenylate cyclase (Cya)	Calmodulin-activated adenylate cyclase and pore-forming cytolysin	*cya*	Chromosome	*B. pertussis, B. parapertussis, B. bronchiseptica*

(*continued*)

Table 12-1. **Properties of selected bacterial toxins (*continued*)**

TOXIN NAME	BIOLOGICAL ACTIVITY	GENE NAME	GENE LOCATION	BACTERIA THAT PRODUCE THE TOXINS
Multisubunit AB-type toxins				
AB$_5$-Type Toxins				
Cholera toxin (CT)	G-protein modifying ADP-ribosyltransferase	*ctxAB*	Phage	*Vibrio cholerae, Vibrio mimicus*
Heat-labile enterotoxin (HLT)		*eltAB, etxAB*	Plasmid, chromosome	*E. coli*
Pertussis toxin (PT)		*ptxA–E*	Chromosome	*B. pertussis*
AB$_2$-Type Toxins				*E. coli, Campylobacter jejuni, Haemophilus ducreyi, Shigella dysenteriae, Helicobacter hepaticus, Aggregatibacter actinomycetemcomitans*
Cytolethal distending toxins			Prophages, plasmids	
CdtA	Binding subunit	*cdtA*		
CdtB	DNA nuclease	*cdtB*		
CdtC	Binding subunit	*cdtC*		
A$_3$B$_7$-Type Toxin				
Anthrax toxins			Plasmid	*Bacillus anthracis*
Protective antigen (PA)	Binding subunit	*pag*		
Edema factor (EF)	Adenylate cyclase	*cya*		
Lethal factor (LF)	Zinc-dependent metalloprotease	*lef*		
Pore-forming toxins				
Staphylococcal leukotoxins, leukocidins	Hexameric membrane channels	*pvl, lukD, lukE, lukF, lukM, lukS*	Chromosome, phage	*Staphylococcus aureus*
Hemolysins	Multimeric membrane channels	*hla, hld, hlg, hly*	Chromosome, plasmid	Many bacterial pathogens
Streptolysin O (SLO)	Heptameric membrane channels	*slo*	Chromosome, plasmid	*Streptococcus pyogenes*
Streptolysin S (SLS)	Large multimeric membrane channels	*sls*	Chromosome	*S. pyogenes*
Perfringolysin O (PFO)	Multimeric membrane channels	*pfo*	Chromosome	*Clostridium perfringens*
Listeriolysin O (LLO)	Multimeric membrane channels	*hly*	Chromosome	*Listeria monocytogenes*
Extracellular proteins, exoenzymes, and peptides				
Staphylococcal	Serine protease		Chromosome	
Exfoliating toxins		*eta, etb, etc, etd* *exhA, exhB, exhC, exhD*		*S. aureus* *Staphylococcus hyicus*
Superantigens	Superantigen (Sag)		Phage, prophages, chromosome, plasmid	
Staphylococcal				
Superantigen		*sea*		*S. aureus*
Enterotoxins		*sed, sej, seg, sen, sei, sem, seo, sel, sec1–3*		
Toxic shock syndrome toxin (TSST)		*tst*		
Streptococcal				
Superantigen		*sea*		*S. pyogenes*
Pyrogenic exotoxins		*speA, speB, speC, speG, speH, speI, speK, speL, speM*		

Table 12-1. Properties of selected bacterial toxins (*continued*)

TOXIN NAME	BIOLOGICAL ACTIVITY	GENE NAME	GENE LOCATION	BACTERIA THAT PRODUCE THE TOXINS
Lipase Toxins	Membrane-disrupting			
Phospholipase A (PLA)				
Lethal toxin	PLA1, PLA2 (ExoU-like)	*RP602, RP534*	Chromosome	*Rickettsia prowazekii*
Phospholipase C (PLC)				
Clostridial	Zinc-dependent PLC	*plc*	Chromosome	*Clostridium perfringens*
α-toxin	PLC	*plc*	Chromosome	*C. novyi, Clostridium haemolyticum*
β-toxin	PLC	*plc*	Chromosome	*C. sordellii, C. novyi*
γ-toxin	PLC	*hly*	Chromosome, plasmid	*C. haemolyticum,* *P. aeruginosa,* *P. aureofaciens*
Listerial				
PI-specific PLC	PlcA	*hylA*	Chromosome	*Listeria monocytogenes*
PC-specific PLC	PlcB	*hlyB*	Chromosome	*L. monocytogenes*
Phospholipase D (PLD)				
PLD	PLD	*pld*	Chromosome	*C. pseudotuberculosis*
Murine toxin		*ymt*	Plasmid	*Yersinia pestis*
Sphingomyelinase (SMase)				
β-toxin	SMase	*hlb*	Phage	*S. aureus*
Toxic effector proteins delivered into host cells by bacterial type 3 secretion systems (T3SS)				
Pseudomonas (T3SS)			Chromosome, prophage	*P. aeruginosa*
ExoS	ADP-ribosyltransferase	*exoS*		
ExoT	ADP-ribosyltransferase plus Rho GTPase-activating protein (GAP) domain	*exoT*		
ExoU	Phospholipase A2 (PLA2)	*exoU*		
ExoY	Adenylate cyclase	*exoY*		
Salmonella (T3SS)			Chromosome, prophage, phage	*Salmonella enterica*
SopA	E3 ubiquitin ligase	*sopA*		
SopB	Phosphatidylinositol phosphatase	*sopB*		
SopE	RhoGAP	*sopE*		
SopE2	RhoGAP	*sopE2*		
SipA	Binds actin	*sipA*		
AvrA	Cysteine protease	*avrA*		
SptP	RhoGAP plus tyrosine phosphatase	*sptP*		
SifA	Filament formation along microtubules	*sifA*		
Yersinia (T3SS)			Plasmid	*Y. enterocolitica,* *Y. pestis,* *Y. pseudotuberculosis*
YopE	RhoGAP	*yopE*		
YopH	Phosphotyrosine phosphatase (PTPase)	*yopH*		
YopP/J	Cysteine protease and deubiquitinase	*yopJ*		
YopM	Anti-inflammatory E3 ubiquitin ligase	*yopM*		
YopT	Cysteine protease	*yopT*		
YpkA/YopO	Serine/threonine protein kinase	*ypkA/yopO*		

(*continued*)

Table 12-1. **Properties of selected bacterial toxins (*continued*)**

TOXIN NAME	BIOLOGICAL ACTIVITY	GENE NAME	GENE LOCATION	BACTERIA THAT PRODUCE THE TOXINS
Shigella (T3SS)			Plasmid	*Shigella flexneri*
IpaA	Actin depolymerization	*ipaA*		
IpaB	Membrane pore-forming	*ipaB*		
IpaC	Actin polymerization	*ipaC*		
IpaD	Phosphatidylinositol phosphatase	*ipaD*		
Lipopolysaccharides/bacterial membrane and cell wall components				
Endotoxin (LPS)	Bacterial cell wall component that binds Toll-like receptors	*los, lps* biosynthetic gene cluster	Chromosome	Gram-negative bacteria
Lipoteichoic acid (LTA)	Bacterial cell wall component that binds Toll-like receptors	*lta* biosynthetic gene cluster	Chromosome	Gram-positive bacteria
Peptidoglycan (PG)	Bacterial membrane surface coating component that binds Toll-like receptors	Peptidoglycan (PG) biosynthetic gene cluster	Chromosome	All bacteria
Tracheal cytotoxin (TCT)	Binds Toll-like receptors	LPS biosynthetic gene cluster	Bacterial PG component	*Bordetella* species
Mycolactone	Immunosuppressant and neurosuppressant; pro-apoptotic	*mlsA1, mlsA2, mlsB* biosynthetic genes	Plasmid	*Mycobacterium ulcerans*

The solution to these mysteries regarding the benefit of harboring a toxin to the bacteria undoubtedly lies in realizing that we are almost certainly viewing toxins from the wrong perspective when we assume that they are being produced with the sole aim of damaging the host. Toxins may also play roles in the physiology of the bacteria and/or their viruses. They may regulate cellular or phage functions or bacterial cell-cell signaling, with their impact on the human body being merely an accidental side effect of their action. In some cases, animals other than humans or lower eukaryotes, such as insects or even protozoa or plants, may be the intended targets of these toxins in the environment. This notion would account for why toxins are often so specific for certain types of animal cells and why most toxins are either secreted from the bacterial cells that make them or delivered directly from bacteria into host cells through special secretion systems.

Toxin Characteristics and Nomenclature

As evidenced in Table 12-1, bacterial toxins come in a large variety of forms, ranging from relatively small lipid-like compounds, such as **endotoxin** and the **macrolactones,** to peptides, such as the **superantigens (SAgs),** to large proteins, such as the single-chain DT and the multi-subunit **anthrax toxin (AnTx)**. A term that has sometimes been used to designate the protein toxins of bacteria is **exotoxin**. The word “exotoxin” was chosen to emphasize the fact that the toxins are secreted from the cell into the medium, in contrast to endotoxins, which are components of the bacterial surface and mainly get released through bacterial cell lysis. The term “exotoxin” has been falling out of use because some protein toxins are not secreted but rather accumulate inside the bacterial cell and are released by cell lysis. Others are injected directly into human or animal cells, thereby bypassing the extracellular environment entirely. These toxins are often referred to as **effector proteins** or **exoenzymes** and are directly delivered into the host cell via specialized secretion systems. We will cover secretion systems and delivery of virulence factors in more detail later in chapter 13. For convenience, we will use the word “toxin” to mean all types of virulence factors of bacteria, whether secreted or not, that are toxic to the human or animal host.

Bacterial toxins vary considerably not only in their structures and activities, but also in the host cell types they attack. Names given to different toxins reflect this diversity. Some toxins are simply given letter designations (e.g., **exotoxin A** of *Pseudomonas aeruginosa)*, while other toxins are named for the numerical, Greek, Roman, or alphabetical order in which they were isolated or discovered from a bacterial species, such as the **alpha-**, **beta-**, **gamma-**, **delta-**, and **epsilon-toxins** produced by *Clostridium perfringens*. Some toxins are named to indicate what type of host cells they

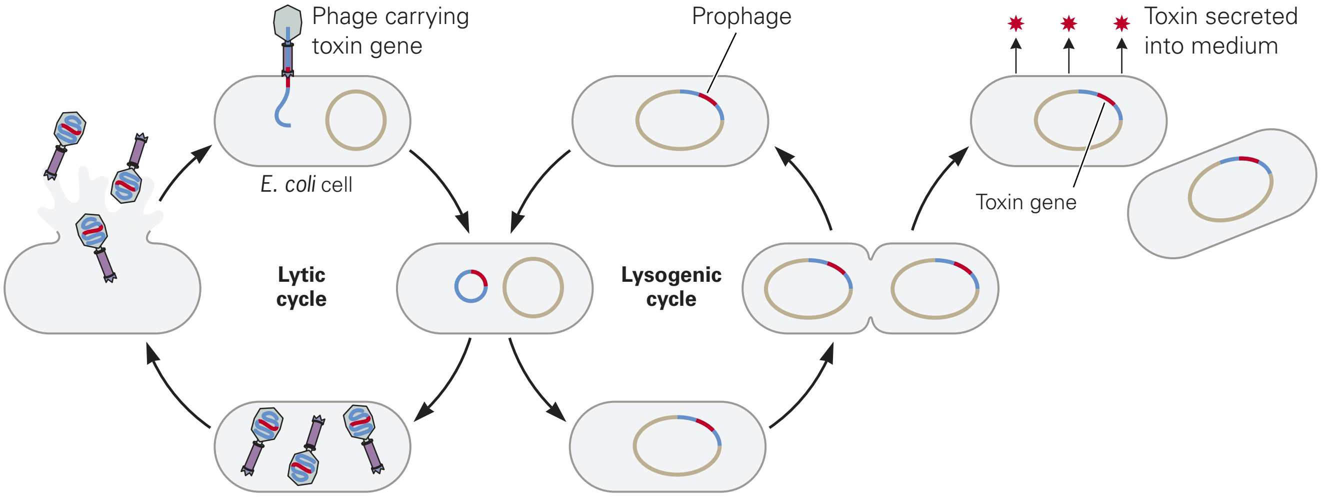


Figure 12-1. Toxin-encoding bacteriophage. When a bacteriophage delivers its genomic DNA into a bacterium, it can enter a lytic cycle where phage DNA is replicated and phage proteins expressed and assembled into new phage that are then released from the bacterium. Alternatively, if it is a lysogenic bacteriophage, it can integrate into the bacterial chromosome as a prophage, replicating along with the bacterial chromosome until the cell senses stress, at which point the prophage excises from the chromosome and reenters the lytic cycle. If the prophage remains in the chromosome long enough, it will eventually mutate to the point that it can no longer excise. If the prophage encodes a toxin gene, has a promoter for the toxin gene, or inserts into a region of the chromosome with a promoter, then the bacterium becomes capable of producing the toxin, which can be secreted or released through bacterial cell lysis.

attack. Toxins that attack a variety of different cell types are often called **cytotoxins**, whereas toxins that attack specific cell types may be designated according to the cell type or organ affected (e.g., **neurotoxin** attacks nerve cells; **leukotoxin** attacks leukocytes; **hepatotoxin** attacks liver cells; **cardiotoxin** attacks heart cells; **verotoxin** attacks Vero cells, which are a green monkey kidney cell line frequently used in toxicity assays; and **enterotoxin** attacks intestinal mucosal or enteric cells). A frequent source of confusion for many students is the term "enterotoxin," which should not be mistaken for "exotoxin." Enterotoxin is a specific term that denotes a toxin that attacks the GI tract and causes diarrhea or vomiting (i.e., causes enteric symptoms).

Toxins are sometimes named for the bacterial species that produces them or for the disease with which they are associated. For example, **cholera toxin (CT)**, produced by *Vibrio cholerae*, is the cause of cholera (watery diarrhea). **Shiga toxin (STx)**, produced by *Shigella dysenteriae*, causes bacterial dysentery (bloody diarrhea), which was first described in 1897 by the Japanese bacteriologist Kiyoshi Shiga (1871–1957). *Bacillus anthracis* secretes AnTx, a multisubunit toxin that is responsible for lethal anthrax disease. *C. diphtheriae* secretes **DT**, which causes the disease diphtheria (respiratory and heart failure). The **tetanus neurotoxin (TeNT)** is produced by *Clostridium tetani* and causes tetanus (lockjaw, spastic paralysis), while variants of BoNT are produced by different strains of *C. botulinum* and causes botulism (flaccid paralysis). The BoNT variants are further designated by letters, based on their order of discovery and immunological cross-reactivity, into seven major serotypes (A through G). In this case, antibodies generated against one serotype, say BoNT/A, do not bind to or neutralize the other neurotoxin serotypes, BoNT/B through BoNT/G. Variants that are produced by different bacterial isolates often have sequence differences, but are still cross-reactive with serotype-specific antibodies. These variants are labeled for serotype and then numbered. For example, BoNT/A1 and BoNT/A2 can both be recognized and neutralized by the same antibodies, but other BoNTs cannot.

Toxins can also be named on the basis of their enzymatic or cellular activities (e.g., **adenylate cyclase** (**Cya**), a toxin produced by *Bordetella pertussis*, the cause of whooping cough; **lecithinase**, a toxin produced by *C. perfringens,* a cause of gangrene, with phospholipase activity that cleaves lecithin phospholipid molecules; **multifunctional auto-processing repeats-in-toxin (MARTX) toxin**, a family of related large toxins produced by *Vibrio vulnificus* and other *Vibrio* species, which are comprised of multiple domains with different biological activities; the **exfoliative toxins** from *Staphylococcus aureus* that cause scalded skin syndrome, characterized by loss of the outermost layer of the skin (exfoliation); the **vacuolating toxin (VacA)** from *Helicobacter pylori* that forms large intracellular vacuoles in intoxicated target cells; and **hemolysin (Hly)**, a large family of membrane pore-forming toxins produced by many Gram-negative and Gram-positive pathogens that disrupt cell membranes and/or lyse red blood cells to release hemoglobin). Still other toxins are named for their biochemical properties, such as the **heat-labile toxin** (**HLT**) and **heat-stable toxins** (**HST**) from *E. coli* that are respectively sensitive and resistant to heat treatment.

Some toxins have more than one name. For example, a toxin produced by *E. coli* O157:H7 (the "killer *E. coli*") has been called **Shiga-like toxin (SLT)**, because it is closely related to the STx produced by *Shigella* species, or verotoxin, because it is toxic for Vero cells. An exotoxin produced by *C. perfringens* is called both α-toxin and lecithinase. A potent toxin produced by *Pasteurella multocida* has been variously called osteolytic toxin, because it causes bone degradation; dermonecrotic toxin, because it causes necrotic skin lesions; mitogenic toxin, because of its proliferative effects on certain tissues or cells; and G-protein-deamidating toxin, because it deamidates glutamine side-chain groups at the active sites of G proteins, as well as simply ***Pasteurella multocida* toxin (PMT)**.

To add another layer to the nomenclature nightmare of toxin designation, toxins are often designated as Types I-III toxins, based on their mechanism of action. Normally, the first step in toxin action is binding to the target cell. This binding step may be followed by direct extracellular action on a cell surface component (Types I and II) or by internalization of the toxin and action on an intracellular target (Type III). **Type I toxins** bind to the target cell surface, but they are not translocated into the target cell, and instead they act extracellularly. An example of this type of toxin are the SAgs, which bind to surface MHCII molecules on antigen-presenting cells (APCs) and T cell receptors (TCRs), forcing the MHCII and TCR complexes into unnatural interactions that induce the T cells to proliferate and produce copious amounts of pro-inflammatory cytokines, hence the designation as "superantigen" (see the following). **Type II toxins** also act extracellularly on eukaryotic cell membranes (e.g., phospholipases, lecithinases, and pore-forming cytotoxins) and exert their effect by destroying the integrity of the mammalian cell membrane, often lysing the cell in the process. **Type III toxins** are the classical **A-B type toxins**, which have two functional components, an enzymatic component or domain (denoted as A) that activates or inactivates some intracellular target or signaling pathway to cause its toxic effect on the eukaryotic cell and a binding component or domain (denoted as B) that recognizes a specific receptor on the host cell and mediates delivery of the A portion into the cell cytosol. A-B type toxins come in two main "flavors", single-chain peptides with multiple domains, such as DT and the BoNTs, and multi-subunit complexes, such as CT and AnTxs. The single-chain A-B toxins often have an additional functional domain, denoted as the **T domain** (for **translocation**), which mediates transport of the A domain across the endocytic vesicle membrane and into the cytosol. In the following sections, we consider examples of each of the different types of bacterial toxins.

Nonprotein Toxins

Endotoxins. Chapter 3 describes the steps in the development of shock during bacterial infection of the blood in response to the presence of integral bacterial membrane components, such as **lipopolysaccharide (LPS)** or **lipoteichoic acid (LTA)**, or fragments of the bacterial **peptidoglycan (PG)** cell wall (Figure 12-2). These **endotoxins** are released into the surrounding medium when bacteria lyse or turn over their cell walls. Bacterial surface components and fragments in the blood trigger widespread, excessive pro-inflammatory cytokine production, which leads to symptoms of shock. LPS endotoxin produced by Gram-negative bacteria, such as *E. coli*, consists of a polysaccharide (sugar) chain (**O antigen**) that is attached to a negatively charged polysaccharide core, which is attached to a hydrophobic lipid moiety, known as **lipid A** (see Figure 12-2). LPS forms the outside leaflet of the outer membrane of Gram-negative bacteria. The polysaccharide O-antigen chain is highly variable among different bacteria and elicits antibody production. In contrast, toxicity is strongly associated with the hydrophobic lipid A component. LPS acts as a prototypical endotoxin through its binding to LPS-binding protein (LBP), which interacts with Toll-like receptors (TLRs) on the cell surfaces of host macrophages, dendritic cells, and neutrophils (see chapter 3). This binding promotes secretion of pro-inflammatory cytokines (e.g., IL-1, IL-6, IL-8, TNF-α, and platelet-activating factor) and nitric oxide, resulting in **endotoxic shock** (also called **septic shock**).

LPS bound to LBP interacts with surface signaling complexes of CD14 receptors, signal-transducing TLRs (mainly TLR4 for most enteric bacteria, but sometimes TLR2/TLR6 for certain pathogens that have modified lipid A cores), and the adaptor protein MD-2 that associates with TLR4 and is required for optimal TLR-mediated signaling induced by LPS (see Figure 3-6). LPS-induced cytokine release causes activation of the alternative complement cascade, increased vascular permeability, and activation of the coagulation cascade. This in turn causes activation of Factor XII and formation of blood clots in small vessels, known as **disseminated intravascular coagulation** (**DIC**), resulting in an overall decrease in blood pressure and blood supply to critical organs. This ultimately results in multiple organ system failure and accumulation of fluid in lungs, called **acute respiratory distress syndrome** (**ARDS**) (see Figure 3-17).

Unfortunately, treatment of a systemic bacterial infection with antibiotics, which can cause the lysis of bacterial cells and release of even more cell surface components, such as LPS, sometimes makes the disease worse. For example, treatment of syphilis, a sexually transmitted disease caused by the spirochete *Treponema pallidum*, with certain antibiotics like penicillin can result in the Jarisch-Herxheimer reaction (also known as lesions of syphilis) due to the acute

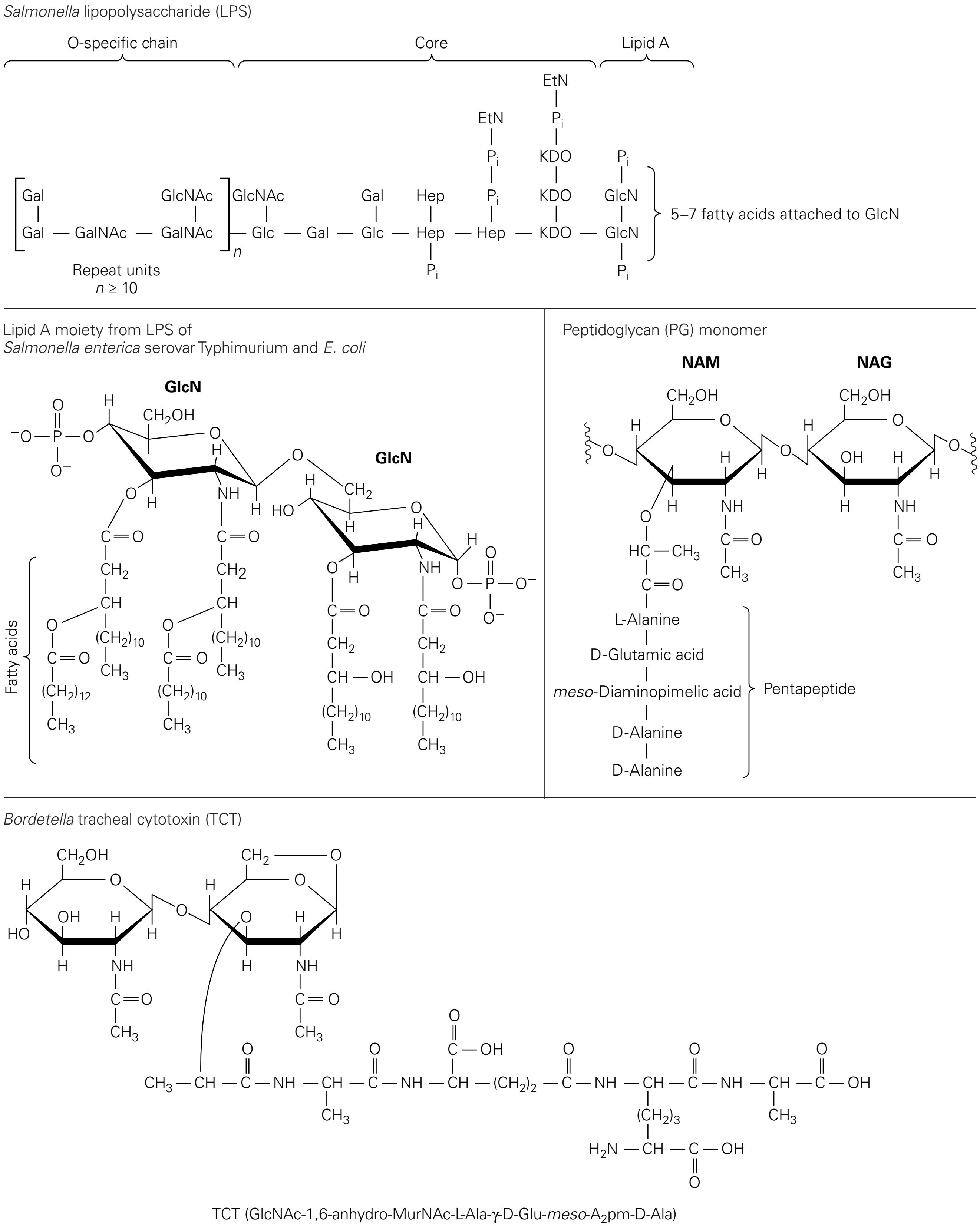

Salmonella lipopolysaccharide (LPS)
O-specific chain
Core
Lipid A
Gal
Gal — GalNAc — GalNAc
GlcNAc
n
Repeat units
n ≥ 10
GlcNAc
Glc — Gal — Glc — Hep — Hep — KDO — GlcN
Gal
Hep
Pi
EtN
KDO
GlcN
5–7 fatty acids attached to GlcN
Lipid A moiety from LPS of
Salmonella enterica serovar Typhimurium and E. coli
GlcN
GlcN
Fatty acids
Peptidoglycan (PG) monomer
NAM
NAG
L-Alanine
D-Glutamic acid
meso-Diaminopimelic acid
D-Alanine
D-Alanine
Pentapeptide
Bordetella tracheal cytotoxin (TCT)
TCT (GlcNAc-1,6-anhydro-MurNAc-L-Ala-γ-D-Glu-meso-A2pm-D-Ala)

Figure 12-2. Examples of endotoxins. Shown are the structures of lipopolysaccharide (LPS) from the outer membrane of *Salmonella*, including the toxic hydrophobic endotoxins (lipid A) common to both *Salmonella* and *E. coli*, a muropeptide fragment of the peptidoglycan (PG) cell wall, and the *Bordetella* tracheal cytotoxin, (TCT), which is also a fragment from the PG cell wall of *Bordetella*. P_i, phosphate group; EtN, ethanolamine; Hep, l-glycero-d-mannoheptose; KDO, 2-keto-3-deoxy-d-mannooctulosonic acid; A_2pm, diaminopimelic acid.

increase in the release of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α). Similar reactions occur from antibiotic treatment of infection with other spirochetes, including borreliosis (Lyme disease from *Borrelia burgdorferi* or tick-borne relapsing fever from *Borrelia recurrentis*) and leptospirosis (Weil's disease or severe pulmonary hemorrhagic syndrome from *Leptospira* species).

Certain pathogens, such as *B. pertussis*, the causative agent of **pertussis** (**whooping cough**), release a low molecular-weight glycopeptide derived from the PG. This toxin, called **tracheal cytotoxin** (**TCT**) (Figure 12-2), is responsible for the respiratory cytopathology observed during whooping cough, including ciliostasis (arrest of ciliary movement), specific extrusion of ciliated cells from the respiratory epithelium, and release of the inflammatory cytokine IL-1. Without ciliary activity, coughing becomes the only way to clear the airways of accumulating mucus, bacteria, and inflammatory debris. TCT thereby triggers the violent paroxysmal coughing episodes symptomatic of pertussis. In addition, the absence of ciliary clearance predisposes the patient to secondary pulmonary infections, which are the main cause of pertussis mortality. How the cough persists for months after clearance of the bacteria and toxins from the body is still a mystery.

Mycolactone Toxins. In contrast to LPS endotoxin, other lipid-like toxins, such as the polyketide-derived **mycolactones**, have both cytotoxic and immunosuppressive properties. *Mycobacterium ulcerans* is an emerging human pathogen harbored by aquatic insects. It is the causative agent of **Buruli ulcer**, a devastating skin disease rife throughout tropical and subtropical regions of Central and West Africa, Central and South America, Asia, and Australia. *M. ulcerans* strains make a variety of mycolactones, which are polyketide-derived macrolides that are made as secondary metabolites. Infection with *M. ulcerans* causes progressive necrotic lesions that, if left untreated, can extend to cover up to 15% of a patient's body surface and can lead to lifelong disability and occasionally death. The most remarkable feature of the disease is that even at advanced stages, there is very little inflammation and no physical pain, a property attributed to the immunosuppressive properties of the mycolactone.

The emergence of *M. ulcerans* as a pathogen most likely reflects the acquisition of a large 174-kilobase plasmid (pMUM001) that is not found in the closely related opportunistic aquatic pathogen *Mycobacterium marinum*. The plasmid encodes a cluster of genes encoding three giant **polyketide synthases** (*mlsA1*, *mlsA2*, and *mlsB*) (Figure 12-3). The modules responsible for building the polyketide structural extensions appear to be functionally similar since there is a very high level of sequence identity among the different activity domains of the MLS cluster (>97% amino acid identity).

Peptide and Protein Exotoxins

Superantigens (Type I Toxins). In the late 1970s, a new and frightening disease appeared in young women, a disease called **toxic shock syndrome** because the afflicted women developed shock-like symptoms. Some of the women even died. As more was learned about toxic shock syndrome, it became clear that this was not shock caused by Gram-negative or Gram-positive bacteria in the bloodstream, but rather by a toxin produced by the Gram-positive bacterium *S. aureus* that colonized the vagina and produced the toxin there. The toxin proved to be an SAg. The reason the disease was seen only in women was that it was associated with a type of superabsorbent tampon that could be left in place for extended periods of time. The bacteria were growing in the tampon, where conditions were conducive to toxin production, and then entered the bloodstream and spread throughout the body. Not all strains of *S. aureus* produce this SAg, accounting for why there were not more cases of the disease. The superabsorbent tampons were soon taken off the market, and the occurrence of toxic shock syndrome has decreased significantly since the 1980s.

Toxic shock syndrome is still of interest, however, because it shows that protein toxins can cause a type of shock that is very like the shock induced by circulating LPS or LTA. Although **toxic shock syndrome toxin** (**TSST**) has properties very different from those of LPS or LTA, its ability to force unnatural associations between macrophages and T cells, the hallmark of an SAg, causes an outpouring of cytokines that trigger the shock process. In addition, SAg molecules appear to act synergistically with LPS to increase the ability of LPS to elicit cytokine release.

This unusual type of bacterial toxin exerts its effect by binding to the MHCII of macrophages and the receptors on T cells (TCRs) that interact with the MHCII (Figure 12-4). As was discussed in chapter 4, APCs, such as macrophages, normally process antigens by cleaving them into peptides and displaying one of the resulting peptides as a complex with MHCII on the macrophage surface (see Figure 4-6). Only a few helper T cells will have TCRs that recognize this particular MHCII-peptide complex, so only a few T cells will be stimulated (see Figure 4-8). SAgs are not processed by proteolytic digestion inside macrophages, but rather bind directly to MHCII on the macrophage surface. SAgs are classified as Type I toxins because they do not enter the cell. Since this binding to MHCII is general, many different macrophages with or without peptide antigen bound will have SAg molecules bound to their MHCII molecules.

The SAg also binds TCRs, again relatively indiscriminately, and thus forms many more macrophage-T helper cell pairs than would normally form. Thus, instead of a macrophage stimulating 1 in 10,000 T cells (the normal response to an antigen), as many as 1 in 5 T cells can be stimulated by

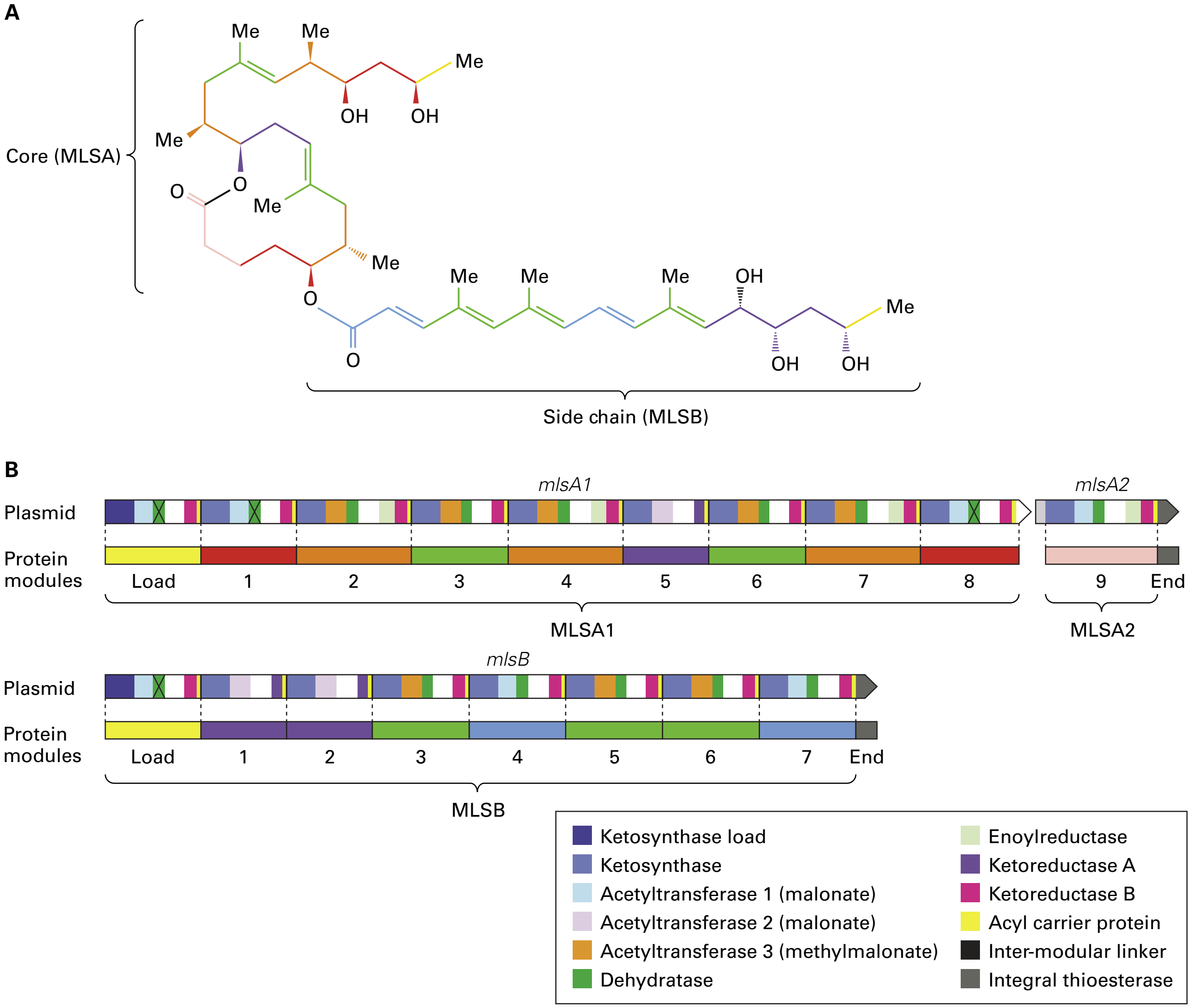

A
Core (MLSA)
Me
Me
Me
OH
OH
Me
O
O
Me
Me
O
O
Me
Me
Me
OH
Me
OH
OH
Side chain (MLSB)
B
mlsA1
mlsA2
Plasmid
Protein modules
Load
1
2
3
4
5
6
7
8
9
End
MLSA1
MLSA2
mlsB
Plasmid
Protein modules
Load
1
2
3
4
5
6
7
End
MLSB
Ketosynthase load
Ketosynthase
Acetyltransferase 1 (malonate)
Acetyltransferase 2 (malonate)
Acetyltransferase 3 (methylmalonate)
Dehydratase
Enoylreductase
Ketoreductase A
Ketoreductase B
Acyl carrier protein
Inter-modular linker
Integral thioesterase

Figure 12-3. The polyketide-derived mycolactone from *Mycobacterium ulcerans* is encoded on a large plasmid. (A) Structure of the mycolactone from *Mycobacterium ulcerans*. **(B)** The 12-membered core structure of the mycolactone is produced by two giant modular polyketide synthases, MLSA1 (1.8 MDa) and MLSA2 (260 kDa), whereas the side chain is synthesized by MLSB (21.2 MDa). Shown is the domain and module organization of the mycolactone PKS genes. Within each of the genes (*mlsA1*, *mlsA2*, and *mlsB*), the color-coded functional modules responsible for building the correspondingly colored polyketide chain extensions (shown in the mycolactone structure in panel A) are made up of different enzymatic activity domains, each designated by a colored activity as indicated in the key. Crossed-out boxes indicate activity domains predicted to be nonfunctional due to presence of an active-site mutation, which is confirmed by the lack of that corresponding modification in the mycolactone structure. Note the highly repetitive nature of each of the modules and the structural outcomes from each of the activities within the corresponding functional modules. Adapted from Stinear TP, Mve-Obiang A, Small PL, Frigui W, Pryor MJ, Brosch R, Jenkin GA, Johnson PD, Davies JK, Lee RE, Adusumilli S, Garnier T, Haydock SF, Leadlay PF, Cole ST. 2004. *Proc Natl Acad Sci U S A* **101:**1345–1349, with permission.

the bridging action of SAgs. (This is why they are called superantigens.) Compared to normal antigen-induced T cell responses, where 0.0001% to 0.001% of the body's T cells are activated at any given time, SAgs can activate up to 20% of the total T cell population. When helper T cells are stimulated by macrophages, one result is that the T cells release massive amounts of pro-inflammatory cytokines, especially interleukin-2 (IL-2). SAg action thus causes excessively high levels of IL-2 to circulate in the bloodstream, giving rise to a variety of shock symptoms, including nausea, vomiting, malaise, and fever.

In addition to TSST, clinical isolates of *S. aureus* and *Streptococcus pyogenes* produce a wide range of other types of SAgs (see Table 12-1), each with varying sequences,

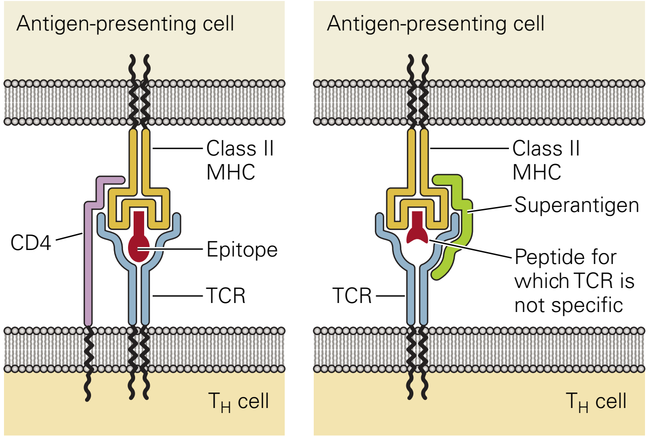


Figure 12-4. Superantigen mechanism of action. Normal interaction of an antigen-presenting cell (APC) and a T cell receptor (TCR) of a T helper (Th) cell (left panel) compared with the interaction of an APC and a TCR mediated by a superantigen (right panel). Superantigens form a bridge complex between the MHCII molecules on APCs and TCRs regardless of whether the correct peptide antigen is present. This tight and long-lasting interaction overstimulates the Th cells to release pro-inflammatory cytokines (especially IL-2), resulting in T cell and B cell proliferation, massive inflammatory responses, and toxic shock.

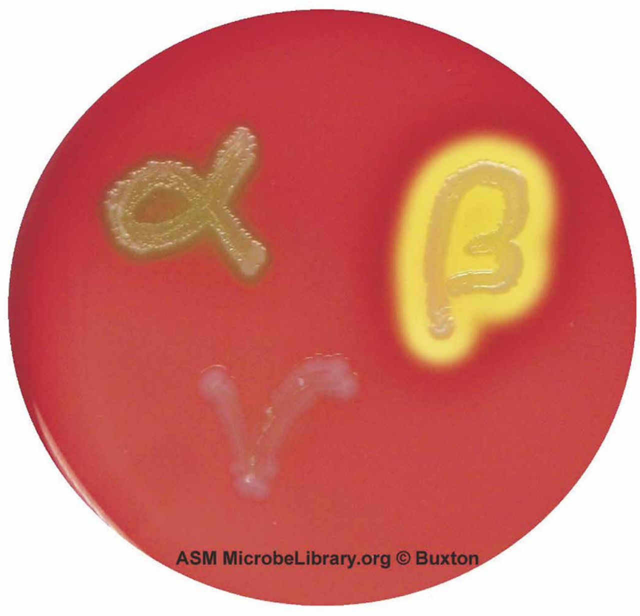


Figure 12-5. Hemolysis. Hemolytic activity is depicted here by observing the appearance of colonies of different strains of streptococci on blood agar plates. In the case of α-hemolysis by *Streptococcus pneumoniae*, the red blood cells remain largely intact, but their pigment is changed from red to green due to H_2O_2 production by the bacteria, which oxidizes the iron in hemoglobin, turning it dark green. In the case of β-hemolysis, the red blood cells surrounding the colony of *Streptococcus pyogenes* are lysed, leaving a cleared yellowish zone. In the case of γ-hemolysis, there is no hemolysis. (This is a misnomer!) Nonhemolytic strains of streptococci rarely cause disease and do not cause hemolysis of red blood cells, so the colonies are whitish-gray and the surrounding agar remains red. Image reproduced from Buxton R. 2005. Blood agar plates and hemolysis: streptococcus and other catalase gram-negative cocci. *ASM MicrobeLibrary.* http://asmscience.org/content/education/imagegallery/image.2881.

potencies, and pathophysiological properties that cause a variety of clinical disorders, including toxic shock, scarlet fever, and mild-to-severe food poisoning. Although most SAgs have similar overall structures comprised of two compact globular domains, they are able to couple to their respective MHCII molecules and TCRs through a number of different ways. The role of SAgs in toxin-mediated disease from staphylococcal and streptococcal infections will be discussed more in chapter 18.

Membrane-Disrupting Toxins (Type II Toxins). Members of a second class of protein exotoxins lyse host cells by disrupting the integrity of the target cell's plasma membranes. These membrane-active toxins are frequently called "hemolysins," because red blood cells are a convenient cell type to use as an assay system (Figure 12-5). However, most of these toxins are able to target a variety of cell types since their target, the cell membrane, is structurally similar among them. As such, the more general term **cytolysin** is also frequently used. Membrane-disrupting toxins have two different ways by which they can mediate similar virulence outcomes. In some cases, their primary role appears to be outright killing of host cells, especially phagocytes, which serves to release cellular content for nutrient and/or metal acquisition by the bacterium and to evade immune cell recognition and clearance of the bacterium. In other cases, invasive bacteria use membrane-disrupting toxins to escape from the phagosome and enter the cytoplasm before phagolysosomal fusion can occur, which again provides a favorable niche in the cytosol for bacterial proliferation and immune evasion.

There are two main types of membrane-disrupting toxins. One type is a protein that forms channels in the membrane. These **pore-forming toxins** poke holes in membranes by binding to the cell surface, oligomerizing, and inserting into the membrane to form transmembrane channels. Since the osmotic strength of the host cell cytosol is much higher than that of the surrounding environment, the holes formed in the membrane allow an inrush of water into the cell, causing the cell to swell. Because the cell membrane is not strong enough to contain the large influx of liquid, the cell lyses. Pore-forming toxins are further classified by the nature of the peptide structures that form the pores. The α-pore-forming toxins tend to be highly α-helical in their water-soluble state and form pores in membranes using the helices. Examples of this type of pore-forming toxin are **cytolysin A (ClyA)**, **colicin A,** and **colicin Ia** from *E. coli*. The β-pore-forming toxins are rich in β-sheets in their water-soluble state and form a β-barrel in membranes. An example of this type of pore-forming toxin is **α-hemolysin** from *S. aureus*.

The basic steps involved in forming the pores have been known for quite some time and are depicted in Figure 12-6. Interaction with a cell surface receptor is generally required to target the toxin to the host cell. Membrane insertion is often facilitated by acidic pH, which leads to a large conformational change in the protein's tertiary structure to form an insertion-competent intermediate state, followed by membrane penetration. The α-pore-forming toxins do not appear to always require oligomerization to form the membrane pore, but oligomerization appears to be an essential step in pore formation by the β-pore-forming toxins. Most of the A-B toxins (the Type III toxins discussed below) have domains or subunits made up of structural components resembling α- or β-pore-forming toxins, which enable them to penetrate and translocate across host membranes to deliver their toxic components into the cytosol.

The second type of membrane-disrupting toxin is a cytolytic enzyme that compromises the integrity of the membrane by hydrolyzing the membrane phospholipids (Figure 12-7A). Such enzymes go by a variety of names, such as **phospholipase** (phospholipid hydrolyzing), hemolysin (red blood cell lysing), or cytolysin (cell lysing, cytotoxic), but they all act to destabilize the host cell membrane. Some phospholipases remove the charged head group from the lipid portion of the molecule. Because the charged head group stabilizes the lipid bilayer structure of the host cell plasma membrane, removal of this group destabilizes the membrane, and cell lysis results. Other phospholipases cleave at other sites but their end effect is the same.

An example of this type of toxin produced by *C. perfringens* is responsible for an extreme form of wound infection, called **gangrene**. Bacteria producing this toxin create an expanding zone of dead tissue. The skin blackens and may appear swollen and cracked, as if the area had been cooked. The damage can be so severe that the limb has to be amputated. This tissue damage is caused in part by a toxin called **α-toxin** (Figure 12-7B), which hydrolyzes lecithin, a generic term for mammalian cell membrane phospholipids, comprised of a mixture of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidic acid (PA). If this α-toxin enters the bloodstream, it can cause systemic damage to organs, such as the kidney.

A-B Toxins (Type III Toxins). The first protein toxins to be studied in detail at the molecular level were **A-B toxins**. They have therefore formed the standard paradigm for how we think about toxin structure and mechanisms. A-B toxins usually contain two types of functional domains or subunits. The first component (called the A part) confers the toxic (usually enzymatic) activity, while the second component (called the B part) binds to receptors on host cells and helps the A part cross the plasma membrane into the host cell cytosol (an internalization process referred to as translocation), where the A part gains access to its intracellular target. Structures of common types of A-B toxins are illustrated in Figure 12-8.

The simplest type of A-B toxin is synthesized as a single polypeptide with a single binding domain (B) and a single activity domain (A). Frequently, the A and B domains of such toxins are separated during processing of the toxin by a proteolytic cleavage event, although the two functional domain-containing fragments often remain connected by a disulfide bond. The disulfide bond is then broken under the reducing conditions inside the host cytosol. Detachment of the A portion from the B portion is often necessary for the A portion to become enzymatically active. The availability of many crystal structures of single-chain A-B toxins has revealed that most of these toxins have an additional functional domain that previously was considered part of the B domain. This additional domain, which is distinct from the receptor-binding domain (still called the B domain), is referred to as a T domain for its translocation function and is responsible for mediating the transfer of the A domain across the membrane and into the cytosol (more on this later in the chapter).

A more complex type of A-B toxin, a multi-subunit A-B toxin, has an enzymatic subunit (A) that is expressed as a separate polypeptide from the binding subunit (B), which is composed of a complex of multiple subunits, usually pentameric complexes, and so are designated as AB_5 toxins (Figure 12-8B). The B subunits are identical in some cases (e.g., CT), but not in others (e.g., **pertussis toxin** [PT]). In these toxins, the A subunit is attached to the multimeric B subunit complex through noncovalent interactions, which are disrupted when the A subunit is internalized by the host cell. A proteolytic cleavage of the catalytic domain of the A subunit (called the A1 domain) from a tail portion of the A subunit that interacts with the B subunit complex (called the A2 domain) occurs for many of these toxins (e.g., CT, PT).

Both types of A-B toxins bind to cell surface receptors and enter host cells, but their trafficking once inside the cell and the location where they translocate their catalytic domains vary, depending on the nature of the membrane and receptor interactions with the toxin, as illustrated in Figure 12-9. The B domain/subunit binds to a specific host cell surface molecule. Often, the molecule recognized by the B domain/subunit is the carbohydrate portion of a host cell surface glycoprotein or glycolipid, but some B domains/subunits bind to surface proteins, and others bind to both. The receptor-binding domain determines the host cell specificity of the toxin. For example, a toxin whose B

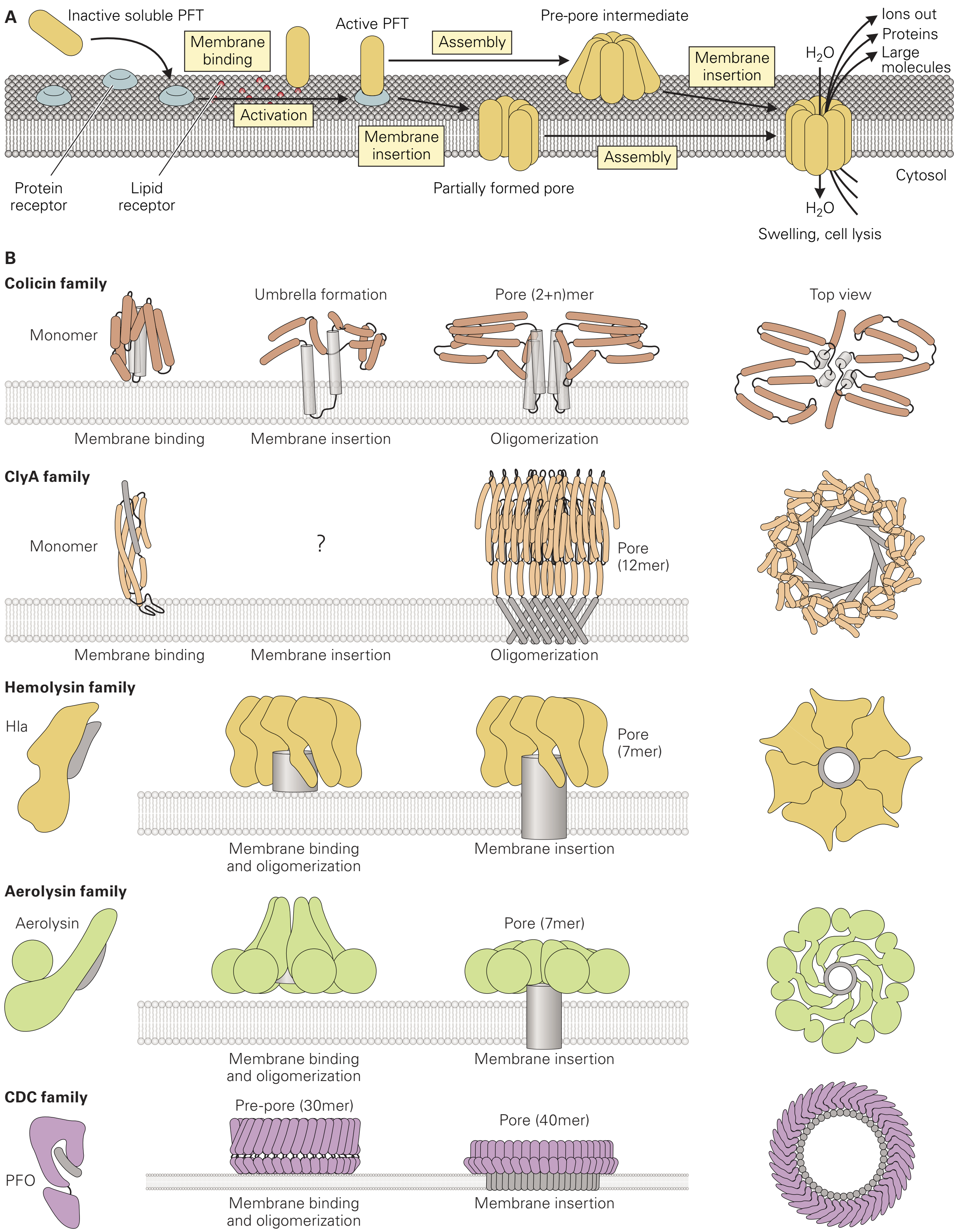

A
Inactive soluble PFT
Membrane binding
Active PFT
Assembly
Pre-pore intermediate
Ions out
Proteins
Large molecules
H_2O
Membrane insertion
Activation
Membrane insertion
Assembly
Protein receptor
Lipid receptor
Partially formed pore
Cytosol
H_2O
Swelling, cell lysis
B
Colicin family
Monomer
Umbrella formation
Pore (2+n)mer
Top view
Membrane binding
Membrane insertion
Oligomerization
ClyA family
Monomer
?
Pore (12mer)
Membrane binding
Membrane insertion
Oligomerization
Hemolysin family
Hla
Pore (7mer)
Membrane binding and oligomerization
Membrane insertion
Aerolysin family
Aerolysin
Pore (7mer)
Membrane binding and oligomerization
Membrane insertion
CDC family
Pre-pore (30mer)
Pore (40mer)
PFO
Membrane binding and oligomerization
Membrane insertion

Figure 12-6. Pore-forming toxins. Pore-forming toxins, also called hemolysins or cytolysins, bind to the host cell surface via a phospholipid or protein receptor that then forms a channel (pore) in the membrane. (**A)** Shown are the steps in toxin pore formation. Once bound to the surface, the toxin monomer either assembles at the surface to form an oligomeric pre-pore intermediate, which then inserts into the membrane (path 1), or first inserts into the host cell membrane and then assembles into oligomers within the membrane to form a pore channel (path 2). Formation of an open pore causes leakage of cell interior components and an inrush of water, leading to cell swelling and possible lysis. (Note: Although the host membrane shown here is the eukaryotic plasma membrane, some pore-forming toxins are also antibacterial and can form pores in the inner membranes of Gram-negative bacteria or the cell membranes of Gram-positive bacteria.) **(B)** Examples of structures and steps in the formation of membrane pores of pore-forming toxin families: colicin A family, cytolysin A family (ClyA), α-hemolysin (Hla) family, aerolysin family, cholesterol-dependent cytolysin family (listeriolysin O, LLO). Adapted from Dal Peraro M, van der Goot FG. 2016. *Nat Rev Microbiol* **14:**77–92, with permission.

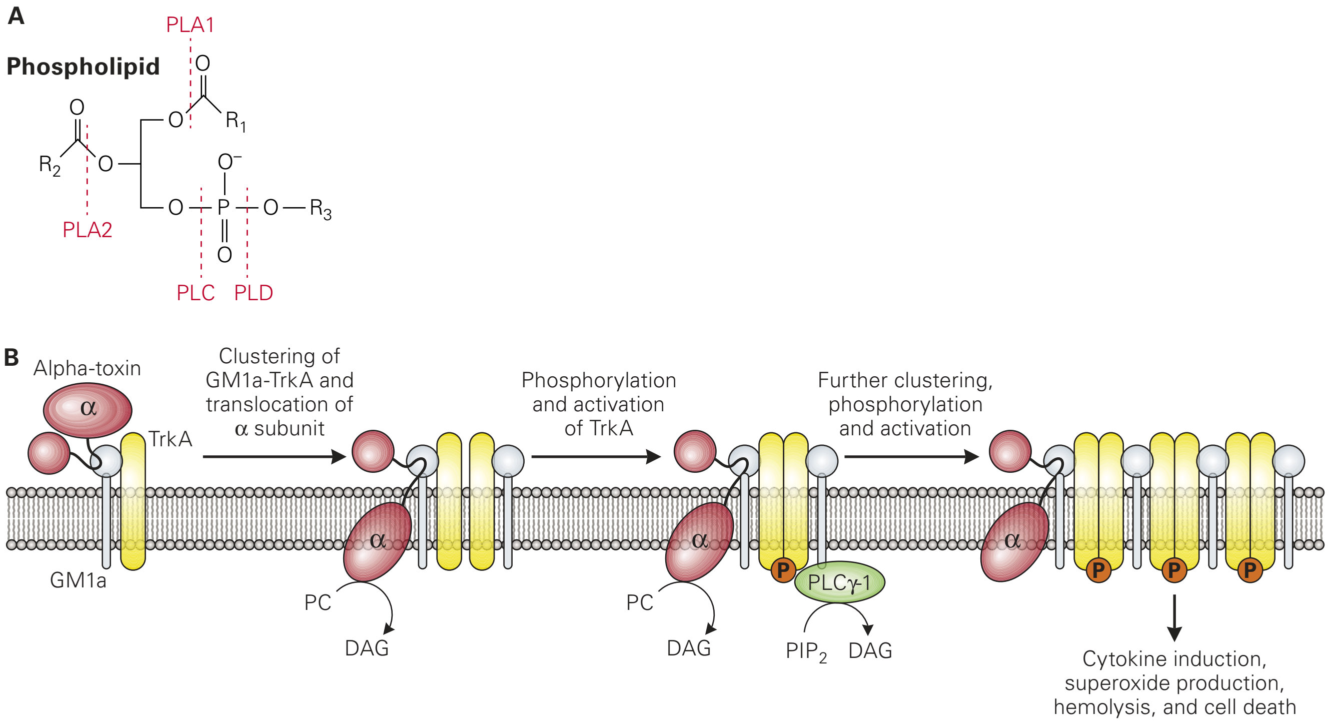

A
Phospholipid
PLA1
O
O
R1
R2
O
O
O⁻
O
P
O
R3
O
PLA2
PLC
PLD
B
Alpha-toxin
α
TrkA
GM1a
Clustering of GM1a-TrkA and translocation of α subunit
α
PC
DAG
Phosphorylation and activation of TrkA
α
PC
DAG
P
PLCγ-1
PIP2
DAG
Further clustering, phosphorylation and activation
α
P
P
P
Cytokine induction, superoxide production, hemolysis, and cell death

Figure 12-7. Membrane-hydrolyzing toxins. (A) Phospholipid-hydrolyzing (phospholipase) types of membrane-disrupting toxins are exoenzymes that act at different sites of the membrane phospholipid to cleave the phospholipid, which destabilizes the membrane bilayer and causes the host cell to lyse. **(B)** *Clostridium perfringens* α-toxin, a key virulence factor contributing to gas gangrene, possesses both phospholipase C (PLC) and sphingomyelinase (SMase) activities. The toxin is comprised of an N-terminal catalytic domain and a C-terminal membrane-binding domain linked by a central loop domain that interacts with membrane gangliosides GM1a. The toxin binds to membranes via GM1a-TrkA complexes and the catalytic domain hydrolyzes the membrane phosopholipids, phosphatidylcholine (PC), phosphatidylinositol (PI), and sphingomyelin (SM). The interactions of α-toxin with TrkA also lead to TrkA phosphorylation, which causes activation of endogenous host cell phospholipases (PLCγ1, PLA2, SMase, and PLD). All of these activities stimulate cellular signal transduction pathways, leading to cytokine induction, superoxide production, hemolysis, and cell death.

domain/subunit binds to a glycoprotein that is found only on the surfaces of neurons will function in the body as a neuron-specific toxin, even though the A domain/subunit has the sort of catalytic activity that would enable it to kill other types of host cells if it could gain entry into their cytoplasm.

After the B domain/subunit binds to the host cell, the whole toxin is taken up into endocytic vesicles and then trafficked intracellularly to a particular cellular compartment, where the A domain/subunit is translocated across the host cell membrane into the cytoplasm. In some cases, acidification of the endosome plays a role in triggering conformational changes that facilitate translocation for those toxins that use this route of entry, first by stimulating the separation of A and B domains/subunits, then insertion of the T domain into the membrane, followed by translocation of the A domain/subunit. Translocation is a complex process that is only beginning to be understood and appears to be different for different toxins. One model posits that the B portion not only binds the host cell surface, but also forms a pore through which the unfolded or partially unfolded A portion threads to gain entry to the host cell cytosol, but this model is somewhat controversial. There is also evidence for host cell proteins that act as chaperones to facilitate the process.

Some multi-subunit A-B toxins have more than one A subunit that can bind to the multimeric B complex. For example, AnTx is an A_3B_7 toxin comprised of two different A subunits, **lethal factor (LF)** and **edema factor (EF)**, that bind in various combinations of three with the B_7 complex made of seven identical B subunits of protective antigen (PA) (Figure 12-10). Unlike the AB_5 toxin complexes of CT and PT that are preformed before interaction with the host cells, the heptameric PA complex first forms on the surface of host cells before binding to the A subunits LF and EF. PA is an 83-kDa protein precursor (PA83) that, once bound to its receptor on the cell surface, gets cleaved by a host protease called **furin** into a 20-kDa fragment (PA20) that is released into the medium and a 63-kDa fragment (PA63) that remains bound to the receptor. The cleavage triggers oligomerization of PA63 on the surface and loss of PA20 exposes a binding site on PA63 for LF and EF. The complex formation

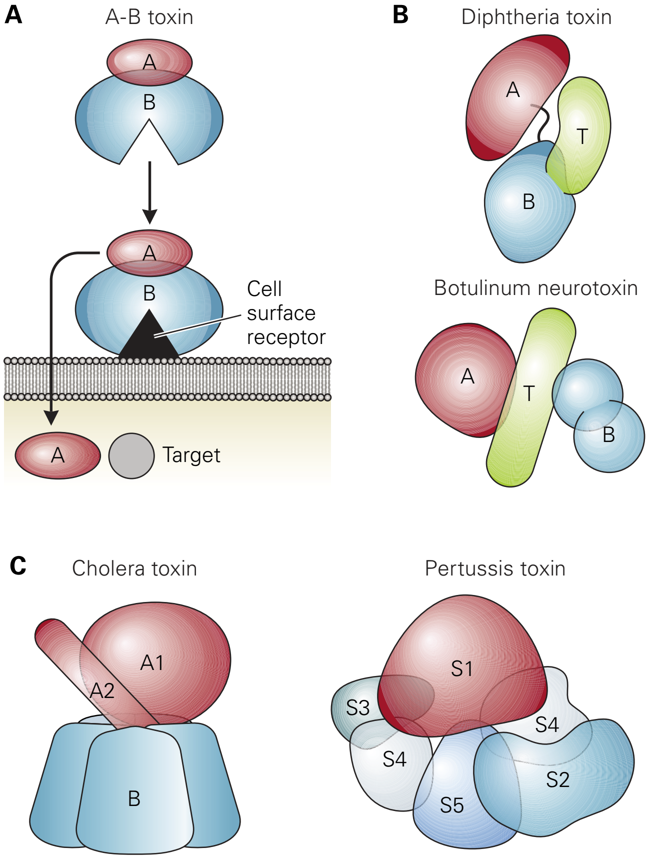


Figure 12-8. Structures of A-B toxins. (A) A-B toxins have two functional domains: a catalytic domain (A domain) that is responsible for toxic activity and a receptor-binding domain (B domain) that is responsible for binding to host cell surface receptors and delivering the A domain into the host cell cytosol. **(B)** Single-chain A-B toxins, such as diphtheria toxin (DT) and botulinum neurotoxins (BoNTs), are synthesized as a single polypeptide chain that is often later processed by proteolytic cleavage into two fragments, one with the catalytic A domain and the other containing the receptor-binding B domain and a translocation T domain. The two fragments are held together by a disulfide bond, which is reduced after reaching the host cytoplasm. In the case of BoNTs, a long peptide belt wraps around the A domain, which follows the same path taken by the substrate, but instead covering the opening of the active site and serving as an inhibitory pseudosubstrate inhibitor of the zinc-dependent protease activity of the toxin A domain. After reduction of the disulfide bond, this belt dissociates and is displaced by the substrate. **(C)** Multi-subunit complex A-B toxins have one or more A subunits and multiple B subunits. Examples of AB_5 toxins are cholera toxin (CT), which has one catalytic A subunit that ADP-ribosylates the α subunit of the Gs type of heterotrimeric GTP-binding proteins and five identical B subunits in its receptor-binding complex (left), and pertussis toxin (PT), which has one catalytic A subunit (named S1) that ADP-ribosylates the α subunit of the Gi and Go types of heterotrimeric GTP-binding proteins and five B subunits (S2-S5), only two of which are the same in its receptor-binding complex (right).

triggers toxin uptake into endocytic vesicles, where acidification then results in translocation of LF and EF into the cytosol through a pore formed by the PA63 oligomeric complex.

Other toxins, such as the AB_5 toxins CT and STx, appear to use alternative routes for gaining entry into the cytosol that do not involve endosomal acidification. These toxins instead undergo retrograde trafficking to the endoplasmic reticulum (ER), where they utilize the pathway normally taken by misfolded proteins to translocate across the ER membrane (Figure 12-11). Misfolded proteins are normally recognized by ER luminal multi-chaperone complexes and transported in an unfolded state across a protein translocation channel, called the **translocon**, which includes the transmembrane protein Sec61 and a cytosolic chaperone p97 with an ATPase activity domain that may help drive the translocation process. The protein is ubiquitinated at lysine residues as it emerges from the channel and targeted for degradation by the proteasome. Following proteolytic cleavage of the toxin A subunit by furin into A1 and A2 and retrograde trafficking to the ER, disulfide bond reduction releases the catalytic A1 domain from the A2-B_5 complex. The A1 protein then associates with the ER chaperones, HEDJ, BiP, and GRP94, and is brought to the translocon channel for translocation into the cytosol. Because A1 does not have lysine residues that can be ubiquitinated, it escapes proteasome degradation and is able to refold into its active form and gain access to its cytosolic 28S rRNA target within the ribosome.

Once the A domain/subunit of A-B toxins has entered the host cell cytosol, it becomes enzymatically active and exerts its toxic effects. Incredibly enough, host cell proteins are now known to aid in the activation of the catalytic domains of some toxins. Although the A component of a given A-B type exotoxin may enter very different cell types, depending on the type of receptors that the B components bind, the effect can be the same or similar since the A domain/subunit catalyzes the same reaction on its cognate substrate targets. For example, the A domain/subunit of a relatively large number of single-chain and multimeric A-B toxins has **ADP-ribosyltransferase** activity (i.e., the A domain/subunit is an enzyme that catalyzes the covalent transfer of the ADP-ribosyl group from the cosubstrate nicotinamide adenine dinucleotide [NAD] to a particular amino acid side-chain group of some host cell protein target [Figure 12-12A]). **ADP-ribosylation** of the host cell protein either inactivates it or causes it to behave abnormally.

The effect of this ADP-ribosylation on the host cell depends on the role of the target protein that is modified. For example, the A domain of DT ADP-ribosylates an unusual posttranslationally modified histidine, called **diphthamide**, of **elongation factor-2 (EF-2)**, a protein that is essential for elongation of the growing peptide chain during host cell

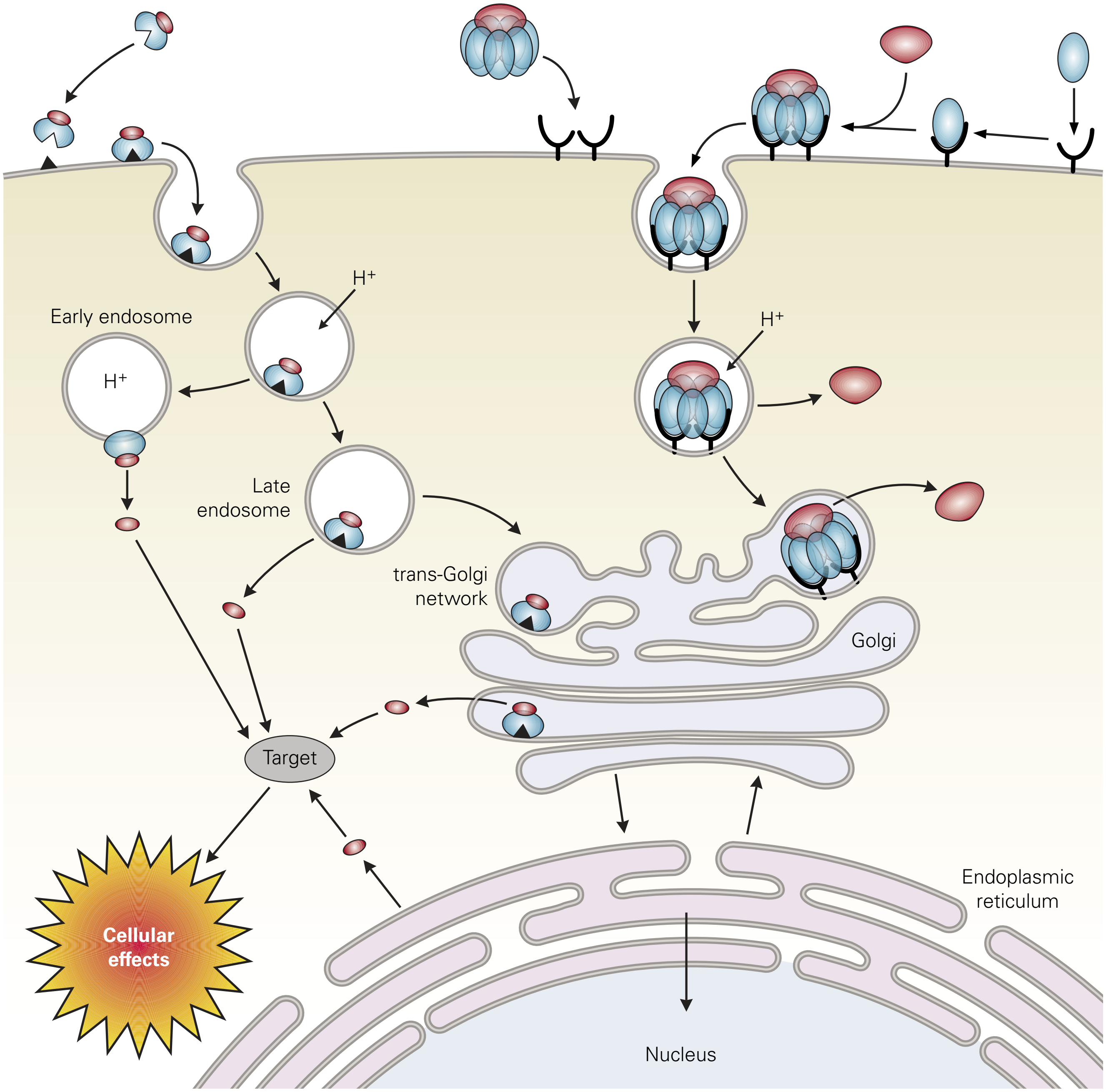


Figure 12-9. Binding and entry of A-B toxins into host cells. There are two main mechanisms for cell entry by A-B toxins. Single-chain A-B toxins (e.g., diphtheria toxin, botulinum neurotoxins) enter via binding to a cell surface receptor (usually a protein or glycoprotein) and their A domains are translocated usually through a pH-dependent processed after endocytosis. Multi-subunit AB_5 toxins (e.g., cholera toxin, pertussis toxin) are pre-assembled at the point of their expression by the bacteria, and the holotoxin AB_5 complex enters host cells by binding to multiple cell surface receptors (usually gangliosides or glycolipids) and their A subunits are translocated either through a pH-dependent process after endocytosis or through retrograde transport to the endoplasmic reticulum (ER), where they are translocated through a misfolded protein secretory transport system into the cytosol. Some toxins even have nuclear localization signal sequences that target them for the nucleus. Toxin A subunits are in red, toxin B subunits are in blue, and the toxin target molecule is gray.

protein synthesis (Figure 12-12B). Thus, the effect of the A domain of DT is to kill the host cell by stopping protein synthesis. In contrast, the A1 subunit of CT ADP-ribosylates a different substrate, namely an arginine residue of the α subunit of the regulatory heterotrimeric GTP-binding protein, G_s. ADP-ribosylation of G_s causes constitutive activation of G_s, which in turn stimulates adenylate cyclase and increases cellular cAMP levels. This causes the host cell to lose con-

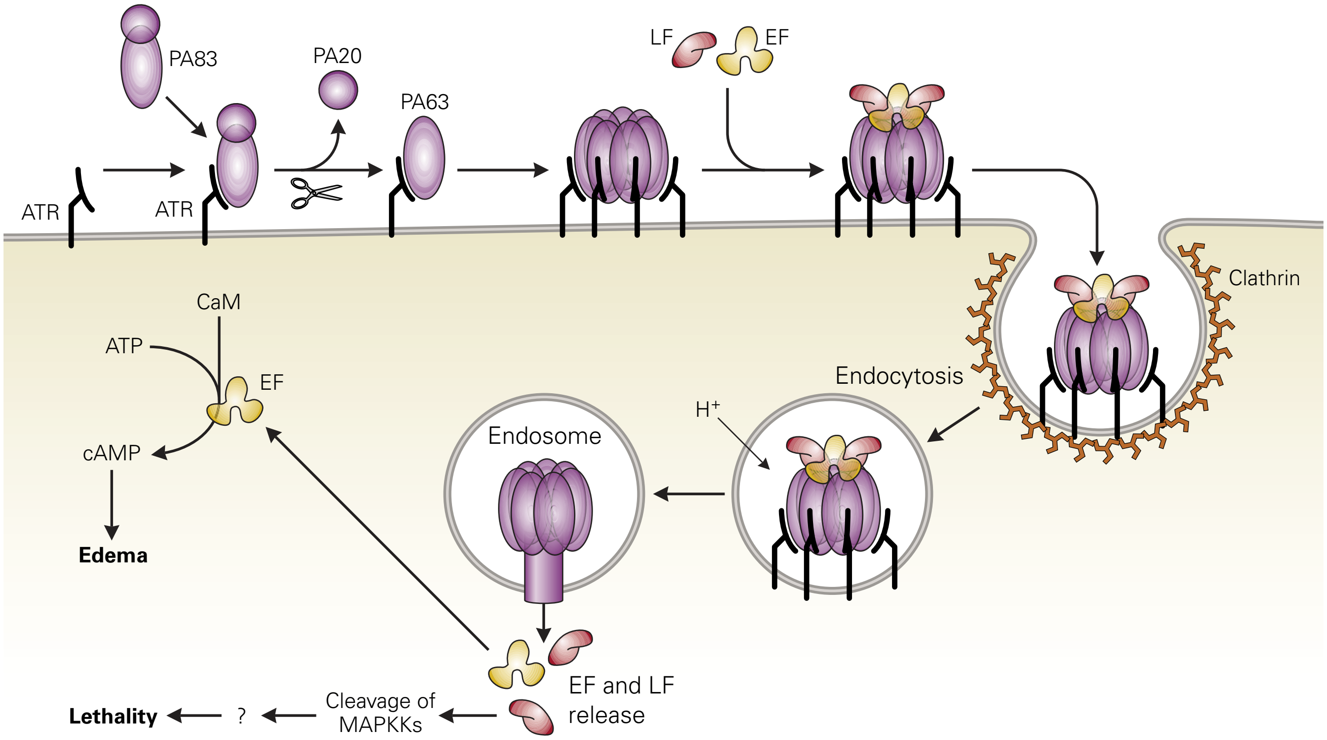


Figure 12-10. Binding and entry of anthrax toxin. The multimeric A_3B_7 toxin, anthrax toxin (AnTx), has two catalytic A subunits, edema factor (EF) (which is an adenylyl cyclase) and lethal factor (LF) (which is a zinc-dependent protease), and seven B subunits called protective antigen (PA83) that must be cleaved before forming a heptameric complex that binds up to three subunits of EF or LF. Unlike other A-B toxins, anthrax toxin subunits are produced and secreted by the bacteria as separate proteins that assemble into the toxin complex on the host cell surface. PA83 first binds to an anthrax toxin receptor (ATR), which can be either tumor endothelium marker-8 (TEM8) or capillary morphogenesis protein 2 (CMG2). A cell surface protease, called furin, cleaves PA83 into PA20 and PA63, triggering PA63 to oligomerize into a heptameric complex known as a pre-pore, which then binds up to three EF or LF subunits and is endocytosed. Acidification of the endosome converts the pre-pore into the translocase pore (channel), through which the EF and LF subunits are translocated into the cytosol.

trol of ion flow and results in a massive loss of host cell water, which is seen macroscopically as diarrhea (more on this later in the chapter).

C. botulinum and *C. perfringens* produce another type of binary ADP-ribosylating toxin (C2 toxin and iota toxin, respectively) that modifies actin (Figure 12-12C). These binary toxins are composed of two nonlinked proteins, a binding and translocation B component and an actin-ADP-ribosylating A component. These toxins modify G-actin at arginine-177, which binds to the ends of F-actin filaments, where it acts to block further binding of G-actin to the F-actin filaments. This prevents actin polymerization and eventually leads to depolymerization of actin filaments and cell rounding.

Not all A-B type toxins have A domains that catalyze ADP-ribosylation of host cell proteins. The catalytic A subunit of STx hydrolyzes the adenine base at a specific nucleotide site of the host cell 28S rRNA molecule in the 60S subunit of the ribosome, resulting in a shutdown of protein synthesis. The catalytic domains of the single-chain BoNTs are zinc-dependent metalloproteases that cleave neuron-specific proteins at specific amino acid sequences, which leads to loss of neurotransmitter release and flaccid paralysis. The catalytic domain of the **cytotoxic necrotizing factors (CNFs)** constitutively activates members of the Rho family of small GTP-binding proteins by deamidating a specific active site glutamine, thereby converting it into a glutamic acid. This results in actin cytoskeletal changes. Examples of selected A-B toxins and their toxic catalytic activities are listed in Table 12-1.

Toxic Effector Proteins of Specialized Secretion Systems

Toxic Effector Proteins. Some toxic bacterial proteins, called **effector proteins**, are directly secreted from the bacterial cytoplasm and injected into the host cell cytoplasm through specialized bacterial secretion systems, such as Type 3 secretion systems (chapter 13), after the bacteria come in direct contact with the host cell. These specialized transport systems are prevalent in Gram-negative bacteria that pos-

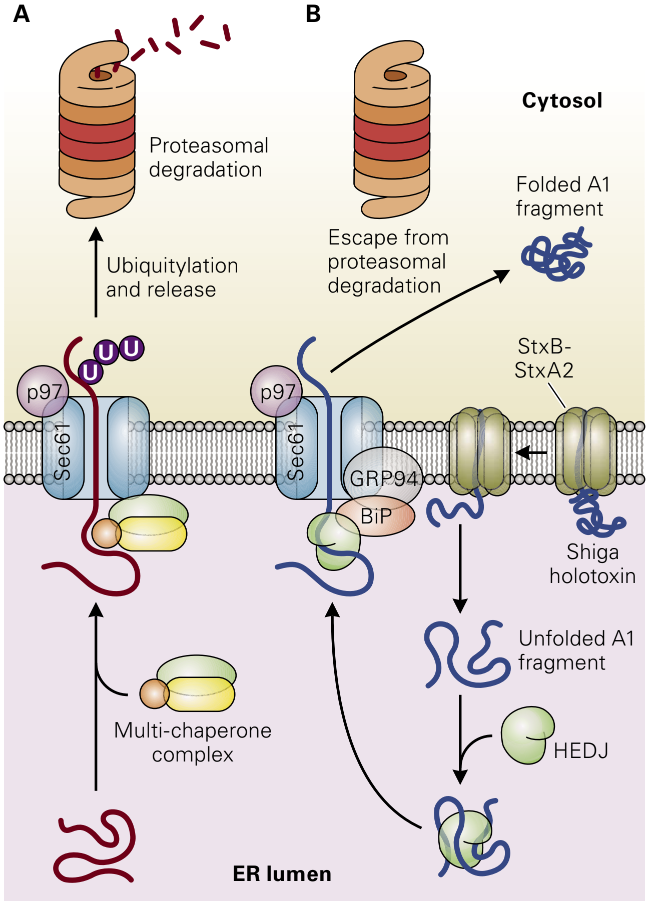


Figure 12-11. Shiga toxin exploits the ER-associated protein degradation system to translocate across the ER membrane into the cytosol. (A) Misfolded proteins are normally recognized by ER luminal multi-chaperone complexes and transported in an unfolded state across a protein translocation channel, called the translocon, which includes the transmembrane protein Sec61 and a cytosolic chaperone p97, which is an ATPase that may help drive the translocation process. The protein is ubiquitinated and targeted for degradation by the proteasome. **(B)** Shiga toxins bind to Gb3 gangliosides on the cell surface and are retrograde trafficked to the ER. Following proteolytic cleavage by furin and subsequent disulfide bond reduction, the catalytic A1 domain is released from the A2-B_5 complex. A1 associates with the ER chaperones HEDJ, BiP, and GRP94 and is brought to the translocon channel for translocation into the cytosol. Because A1 does not have lysine residues that can be ubiquitinated, it escapes proteasome degradation and is able to refold into its active form. Adapted from Johannes L, Römer W. 2010. *Nat Rev Microbiol* **8**:105–116, with permission.

sess an outer membrane and in some Gram-positive that have unusual cell surfaces. These effector proteins were formerly called exoenzymes because they were often found in the culture medium due to leakiness of the secretion system machinery under certain conditions. As indicated by the examples listed in Table 12-1, many of the toxic activities of these effector proteins are very similar to those of A-B toxins and membrane-disrupting phospholipase exotoxins. These effector proteins cause cytopathic or cytotoxic effects on the host cells, and so, in a way, they could be considered the catalytic components of toxins minus the receptor-binding B domain and/or the translocation T domain. Indeed, at one time, it was believed that these toxic proteins were simply “orphan” A subunits, for which their cognate B subunits had not yet been identified. The primary difference is that toxic effector proteins are nontoxic by themselves, such as when they are secreted or released into the medium, and require the specialized secretion system to deliver them into host cells in order to exhibit their toxicity. We will learn more about the delivery of these toxic effector proteins in chapter 13.

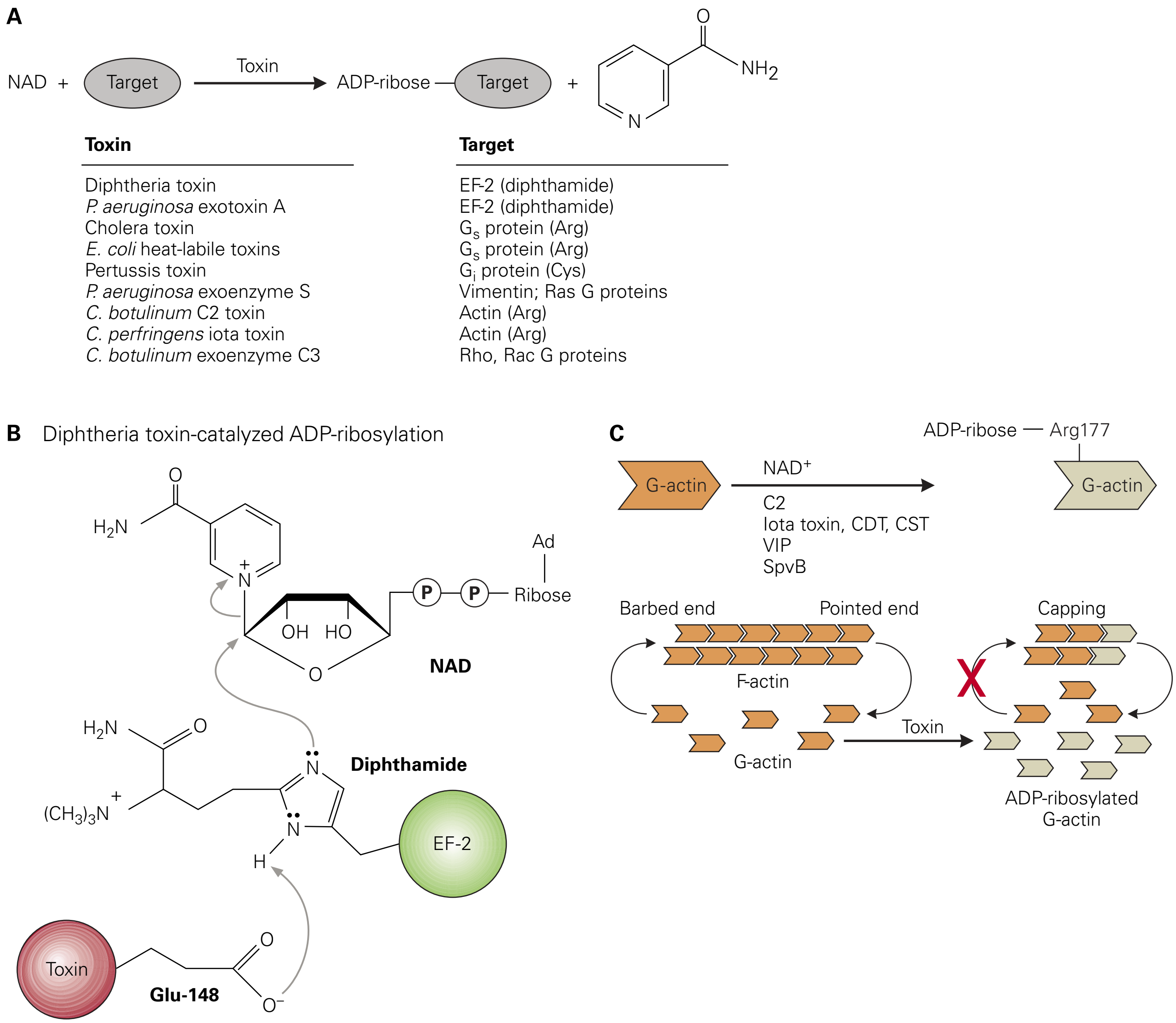
Examples of Toxin-Mediated Diseases

The best way to understand how toxins participate in the disease process is to consider a couple of examples of diseases in which toxins are the primary virulence factor. These examples also illustrate the continuing mystery of why bacteria produce toxins in the first place.

Diphtheria Toxin

DT is one of the best-studied bacterial toxins, and thus serves as a model for understanding how toxins are transmitted, regulated, and produced, as well as how they enter the cytoplasm of eukaryotic cells to elicit their toxic effects. In fact, diphtheria provided the first paradigm for a disease in which the symptoms could be explained entirely by the action of a single molecule, DT. This cause-and-effect relationship helped give rise to the concept of “virulence factor” and ushered in the new era of molecular analysis of bacterial diseases. From that time to the present day, investigations of DT have continued to produce important new insights into toxin structure, function, interaction with host cells, and role in pathogenesis. The tremendous knowledge we now have about DT and its mechanism of action has enabled its exploitation for beneficial purposes. DT has become a prototype for selective targeting and killing of certain cells as a tool for cell and developmental biology research (see chapter 10), for eliminating tumor cells in cancer therapeutics (more on this later in the chapter), and for antigen delivery in vaccine development (see chapter 17).

Diphtheria. The disease diphtheria is caused by infection with *C. diphtheriae*, a Gram-positive, non-spore forming, nonmotile, aerobic rod-shaped bacterium. Diphtheria is normally a disease of children, which can be fatal if not treated. Diphtheria is no longer a serious public health



Toxin	Target
Diphtheria toxin	EF-2 (diphthamide)
P. aeruginosa exotoxin A	EF-2 (diphthamide)
Cholera toxin	G_s protein (Arg)
E. coli heat-labile toxins	G_s protein (Arg)
Pertussis toxin	G_i protein (Cys)
P. aeruginosa exoenzyme S	Vimentin; Ras G proteins
C. botulinum C2 toxin	Actin (Arg)
C. perfringens iota toxin	Actin (Arg)
C. botulinum exoenzyme C3	Rho, Rac G proteins

A
NAD +
Target
Toxin
ADP-ribose
Target
+
O
NH2
N
B Diphtheria toxin-catalyzed ADP-ribosylation
O
H2N
N+
Ad
P
P
Ribose
OH HO
O
NAD
H2N
O
(CH3)3N+
N
Diphthamide
N
H
EF-2
Toxin
Glu-148
O
O−
C
ADP-ribose — Arg177
G-actin
NAD+
C2
Iota toxin, CDT, CST
VIP
SpvB
G-actin
Barbed end
Pointed end
Capping
F-actin
X
Toxin
G-actin
ADP-ribosylated G-actin

Figure 12-12. Toxin-catalyzed ADP-ribosylation of target host proteins. (**A**) The A domain/subunit of a large number of bacterial toxins catalyze the transfer of an ADP-ribosyl group from NAD to a side-chain group of a host cell target protein. Shown are selected examples of ADP-ribosylating toxins. (**B**) Diphtheria toxin-mediated ADP-ribosylation of the diphthamide residue on eukaryotic elongation factor-2 (EF-2) is catalyzed by a glutamic acid at the active site of the A domain, which serves as a base to increase the nucleophilicity of the diphthamide residue on EF-2. Nicotinamide is released as a product during the reaction. **(C)** Clostridial C2-mediated ADP-ribosylation of the arginine residue at position 177 of G-actin. ADP-ribosylated G-actin binds to the barbed ends of F-actin filaments, where it acts as a capping protein to block further addition of G-actin monomers to the F-actin, preventing actin polymerization. The pointed ends are not affected, and so still undergo disassembly. This results in actin depolymerization and cell rounding.

problem in developed countries because infants are routinely vaccinated against it, but diphtheria remains a major cause of childhood mortality worldwide. The diphtheria vaccine is a toxoid version of DT, the "D" in the trivalent DTaP vaccine (more on this in chapter 17). This vaccine has few side effects and confers long-term protection against diphtheria. With periodic booster shots, the vaccine gives lifelong immunity. Unfortunately, there are still many countries where routine vaccination against diphtheria is not feasible for economic reasons. In these countries, diphtheria remains a significant cause of disease and death in infants and children.

Diphtheria starts with colonization of the throat by *C. diphtheriae*. The bacteria are acquired by inhalation of aerosols from an infected person or an asymptomatic carrier. Humans are the only known reservoir of *C. diphtheriae*. The first symptoms are relatively nonspecific: malaise, low-grade fever, sore throat, and loss of appetite. Colonization of the throat causes considerable damage to the mucosal cells due to release of DT that kills the exposed cells. The bacteria remain primarily in the throat and are not usually found in the bloodstream. As the bacterial colonization progresses, a grayish membrane begins to form in the throat and may extend into the lung. The membrane, called a **pseudomembrane**, consists of fibrin, bacteria, and inflammatory cells and adheres to underlying tissue, which bleeds when attempts are made to remove the pseudomembrane. This is a useful sign for diagnosing diphtheria because pseudomembranes caused by other infectious agents are nonadherent. The presence of the pseudomembrane and the bleeding of underlying tissue attest to the fact that bacterial colonization of the throat is causing considerable damage to the mucosal cells. Sudden death can sometimes occur from suffocation when large sections of the pseudomembrane separate and block air passages.

Unlike the colonizing extracellular corynebacteria, DT does enter the bloodstream at lesion sites and causes damage to internal organs, eventually resulting in organ failure. The most serious form of the disease is characterized by irregular heartbeat, difficulty in swallowing, stupor, coma, and finally death, usually from heart or respiratory failure. Although throat colonization by the bacteria is the most common infection route, *C. diphtheriae* can also cause skin infections, which is commonly seen in countries where diphtheria is endemic. Recovery from infection leads to lifelong immunity. In the past, those people who survived childhood usually had acquired immunity to diphtheria by the time they became adults.

Diphtheria Antitoxin. In many diseases where exotoxins are either solely responsible for or make a major contribution to the symptoms of the disease, antibodies to the exotoxin provide effective protection against the disease. Binding of antibodies to the B domain/subunit of the toxin physically interferes with binding of the toxin to its target cell and thus prevents the toxin from entering and exerting its toxic activity. Prior to the era of antibiotics, the only means to treat a toxin-mediated disease such as diphtheria was to neutralize the circulating toxin through injection of a single, massive dose of **antitoxin** (i.e., antibodies against DT usually obtained from horse serum) and hope that the host immune system could then handle the rest. Still today, injection with antitoxin (i.e., **passive immunization**) is an effective means for treating toxin-mediated diseases, including diphtheria and botulism (see the following sections), where the toxins produced by the bacteria cause severe or fatal disease symptoms. For an inspiring account of how a desperate race to deliver diphtheria antitoxin immortalized a dog and started a sport, see Box 12-1.

Diphtheria Vaccine. Before any detailed information was available about toxin structures and mechanisms of action, vaccines were created by a hit-or-miss approach involving genetic mutations, chemical modification, or heat treatment of a toxin to render it nontoxic but still capable of eliciting antibodies that would bind and neutralize the toxin. Such preparations are called toxoids. The highly effective vaccines against diphtheria and tetanus are products of this type of approach (see chapter 17). Today, it is possible to design **toxoid vaccines** in a more rational way. For example, the fact that action of A-B type toxins is dependent on binding of the B domain/subunit to a host cell receptor molecule suggests that the B components, which are not toxic by themselves, would make the best vaccine candidates. Current vaccines against toxin-mediated diseases are based largely on this premise.

It should be noted, however, that immunization with a vaccine based solely on a toxoid only prevents toxin-mediated symptoms; it does not necessarily prevent colonization by the bacterium. For instance, *C. diphtheriae* can still colonize the throats of people who have been immunized against DT. However, no pseudomembrane develops in the throats of these immunized people who are colonized by the bacteria. For this reason, formation of the pseudomembrane is presumed to result from the killing of mucosal cells by DT. It is interesting that in people immunized against DT, the colonizing strain of *C. diphtheriae* usually does not produce toxin.

DT Production. The *tox* gene encoding DT is carried on a group of related **lysogenic corynebacteriophages** (**β-phage** and **ω-phage**). Only bacterial strains infected with these phage produce the toxin (see Figure 12-1), while those infected with another closely related bacteriophage (**γ-phage**) that do not encode the *tox* toxin do not produce DT. The fact that β- and ω-phages can be induced to become lytic, while γ-phage cannot, actually helped facilitate the cloning of the *tox* gene. Isolation and comparison of DNA from each of the three types of phage enabled identification of the DNA segments present in both β- and ω-phages but not in the γ-phage, thereby significantly narrowing down the possible location of the *tox* gene.

Production of toxin by lysogenized *C. diphtheriae* is greatly enhanced when the bacteria are grown in low-iron medium. A DNA-binding repressor protein called **DtxR** (for **diphtheria toxin repressor**), which is related to an iron regulatory protein of *E. coli* called **Fur** (for **ferric uptake regulator**), mediates regulation of the DT gene (Figure 12-13). The fact that expression of the toxin is enhanced when iron levels are low has led to the suggestion that the purpose of

Box 12-1.

A Dog Named Balto, the Iditarod, and Diphtheria Antitoxin…?

On January 21, 1925, Dr. Curtis Welch, the only doctor in Nome, Alaska, diagnosed several Inuit children with diphtheria. He had just enough diphtheria antitoxin to treat up to five patients, but he knew that would not be enough to prevent the rapid spread of the deadly disease throughout the isolated, ice-bound community of 1,400 villagers. Dr. Welch made an emergency call over wireless telegraph to plead for more antitoxin serum. The only antitoxin available turned out to be a small supply of 300,000 units found in a hospital in Anchorage, almost 1,000 miles away (Figure 1). Ice prevented transport via ship and an ongoing blizzard prevented transport via airplane. On January 27, a train was able to bring the antitoxin part of the distance to Nenana, but after that snow and ice prevented further travel. The only choice for delivering the antitoxin was to travel an old dogsled route for mail and supply delivery, called the Iditarod Trail, using 20 relay teams of up to 20 dogs each to travel the remaining 674 miles between Nenana and Nome, which was estimated to take about 13 days. Most of the dog teams traveled relay legs in the range of 18 to 53 miles through blinding artic blizzard winds, ice, and subfreezing (<–40°F) conditions. But particularly perilous trail conditions for one leg of the way forced one dog team, lead by musher Leonhard Seppala and his lead dog Togo, to trek a total of 91 treacherous miles before passing on the antitoxin serum to the next dog team. Ending the entire journey in a record 6 days, the team that covered the last 53-mile leg and delivered the antitoxin in the wee hours of February 2 was mushed by Gunnar Kassen with Balto as his lead dog.

News agencies outside of Alaska learned of Nome's plight from the emergency telegraph message sent by Dr. Welch, and the story of the brave dogsled teams became front-page news all over America at the time. The mushers received special hero medals, and a statue of Balto was erected in Central Park in New York City, where it still stands today to commemorate the heroic race that saved the epidemic-stricken children of Nome (Figure 2). The story of Balto even inspired a popular animated children's movie called *Balto* produced by Steven Spielberg in 1995. The Iditarod Race was revitalized in 1973 and runs the entire 1,150 miles along the Iditarod Trail between Anchorage and Nome. The Iditarod has become one of Alaska's most renowned sporting events, with the best mushers receiving thousands of dollars a year from corporate sponsors.

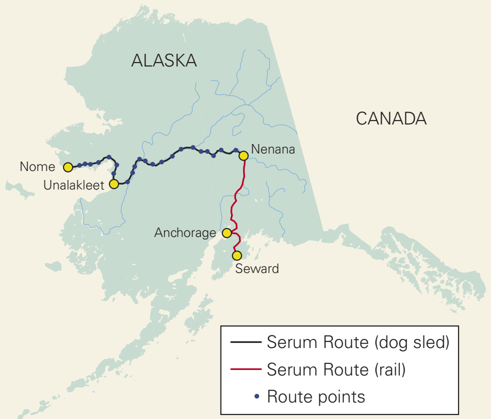

ALASKA
CANADA
Nome
Unalakleet
Nenana
Anchorage
Seward
Serum Route (dog sled)
Serum Route (rail)
• Route points

Figure 1. Map of the route taken in 1925 to deliver diphtheria antitoxin to Nome, Alaska. Adapted from Aboul-Enein BH, Puddy WC, Bowser JE. 2016. *J Med Humanit* doi:10.1007/s10912-016-9428-y, with permission.

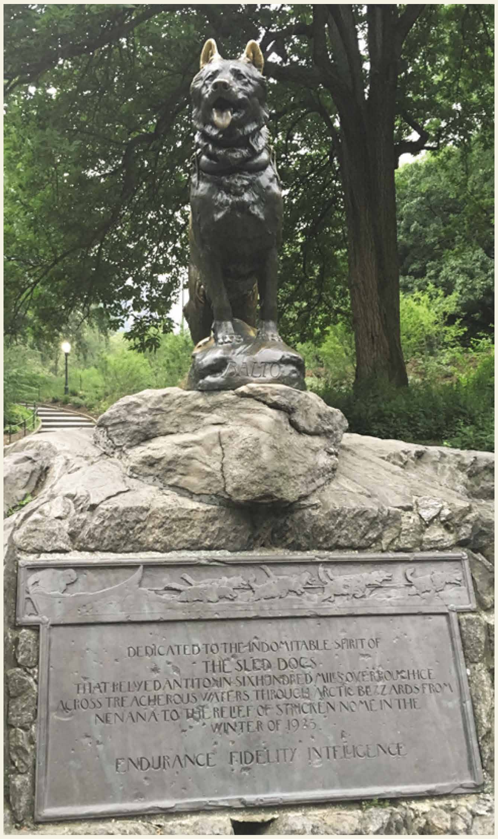

BALTO
DEDICATED TO THE INDOMITABLE SPIRIT OF
THE SLED DOGS
THAT RELAYED ANTITOXIN SIX HUNDRED MILES OVER ROUGH ICE
ACROSS TREACHEROUS WATERS THROUGH ARCTIC BLIZZARDS FROM
NENANA TO THE RELIEF OF STRICKEN NOME IN THE
WINTER OF 1925
ENDURANCE FIDELITY INTELLIGENCE

Figure 2. Statue of Balto with commemorative plaque in Central Park, New York City, New York. Photo courtesy of Yuritzi M. Garcia.

Sources:

The Official Site of the Iditarod: http://www.iditarod.com

Aboul-Enein BH, Puddy WC, Bowser JE. 2016. The 1925 diphtheria antitoxin run to Nome, Alaska: a public health illustration of human-animal collaboration. *J Med Humanit*.

DT may be to kill host cells and thus release iron for use by the bacteria. The only problem with this explanation is that strains that do not produce toxin are able to colonize the human throat just as well as toxin-producing strains. An alternative hypothesis is that because *C. diphtheriae* resides only in human hosts and spread from person to person is essential for its propagation, the symptoms caused by toxin production may increase the spread among people, especially those in close contact or in crowded situations.

Mechanism of DT action. DT is synthesized as a 60.8-kDa precursor in the bacteria that gets further processed into its mature active form in two steps. The initially translated form of DT contains a 25-amino acid leader peptide sequence that is removed by proteolytic cleavage during secretion of the toxin through the bacterial cell membrane by the general secretory (Sec) system (see chapter 13) and DT is released into the extracellular medium as a 58.3-kDa mature polypeptide. After secretion, this polypeptide is further proteolytically cleaved by bacterial proteases in the medium or by the mammalian surface protease furin into an A fragment and a B fragment, which remain joined by a disulfide bond until translocation into the host cell cytosol. Steps involved in toxin binding, endocytic uptake, translocation, and ADP-ribosylation of target EF-2 substrate are shown schematically in Figure 12-14.

The B fragment of DT is actually made up of two domains: one domain called the **R domain** (or B domain), which binds a protein receptor on the host cell surface, and the other called the T domain, which translocates the A domain into the cell cytosol. The DT receptor has been identified as the human-specific **heparin-binding epidermal growth factor-like growth factor (HB-EGF) precursor**. There is also a putative DT receptor accessory protein (DTRAP2) that binds to HP-EGF, but its role in intoxication of DT is not clear. Epidermal growth factors of various types are important signals for cell growth and differentiation. Thus, it is not surprising that the receptor for DT is found on so many different cell types or that its abundance varies so much from one cell type to another.

After the toxin binds to the host cell surface, the toxin is taken up into endocytic vesicles via clathrin-coated pits. Receptor-mediated endocytosis is an important step in toxin action, because the decrease in pH that subsequently occurs in the late endosome makes the translocation process possible. The structure of the T domain of DT resembles that of the colicin 1a family of pore-forming toxins, whose protein conformation is sensitive to acidic conditions. As the pH drops to around 5 in the late endosome, side-chain groups of aspartate and glutamate residues in the loop of helix-loop-helix regions become protonated, making the entire helix-loop-helix more hydrophobic and able to insert into membranes. The change in charge distribution is associated with a change in the conformation of the T domain, which also causes exposure of hydrophobic regions that are normally found in the interior of the toxin and partial unfolding of the A and B domains. The current model posits that partial unfolding of the A and B domains to expose hydrophobic

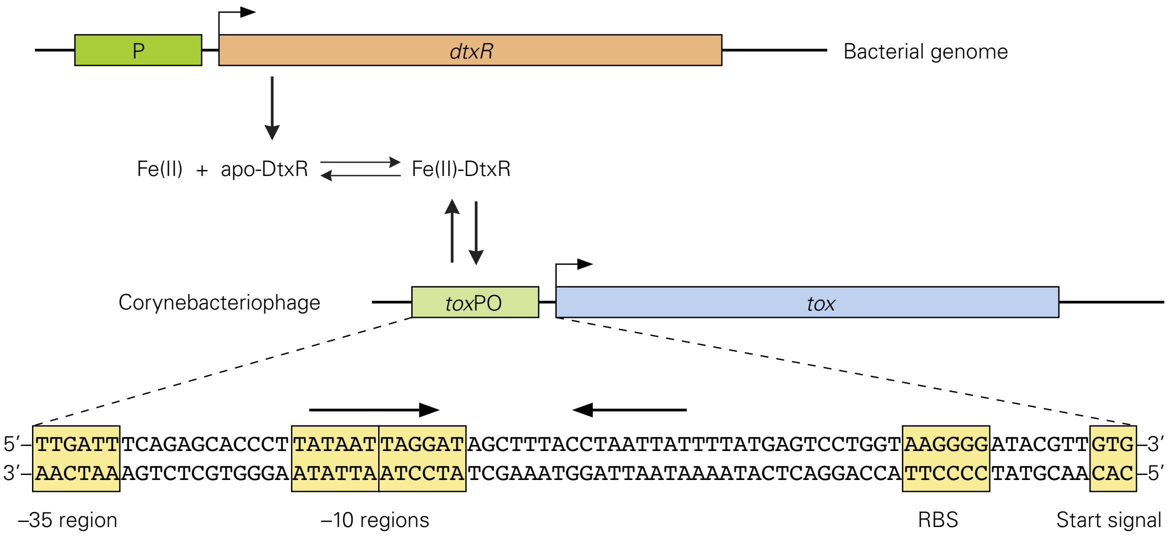

P
dtxR
Bacterial genome
Fe(II) + apo-DtxR
Fe(II)-DtxR
Corynebacteriophage
toxPO
tox
5′–TTGATTTCAGAGCACCCTTATAATTAGGATAGCTTTACCTAATTATTTTATGAGTCCTGGTAAGGGGATACGTTGTG–3′
3′–AACTAAAGTCTCGTGGGAATATTAATCCTATCGAAATGGATTAATAAAATACTCAGGACCATTCCCCTATGCAACAC–5′
–35 region
–10 regions
RBS
Start signal

Figure 12-13. Regulation of DT expression in *Corynebacterium diphtheriae*. The product of a chromosomal gene called *dtxR* regulates the expression of DT. DtxR is a transcriptional repressor that binds to the operator region of the DT *tox* gene only in the presence of ferrous ions (Fe^{2+}). When the levels of iron are high, the Fe(II)-DtxR complex binds the *toxPO* sequence and prevents (represses) transcription. When iron becomes the growth rate-limiting nutrient, which is the prevailing condition within the host, the complex dissociates, allowing transcription to proceed. Adapted from White A, Ding X, vanderSpek JC, Murphy JR, Ringe D. 1998. *Nature* **394:**502–506, with permission.

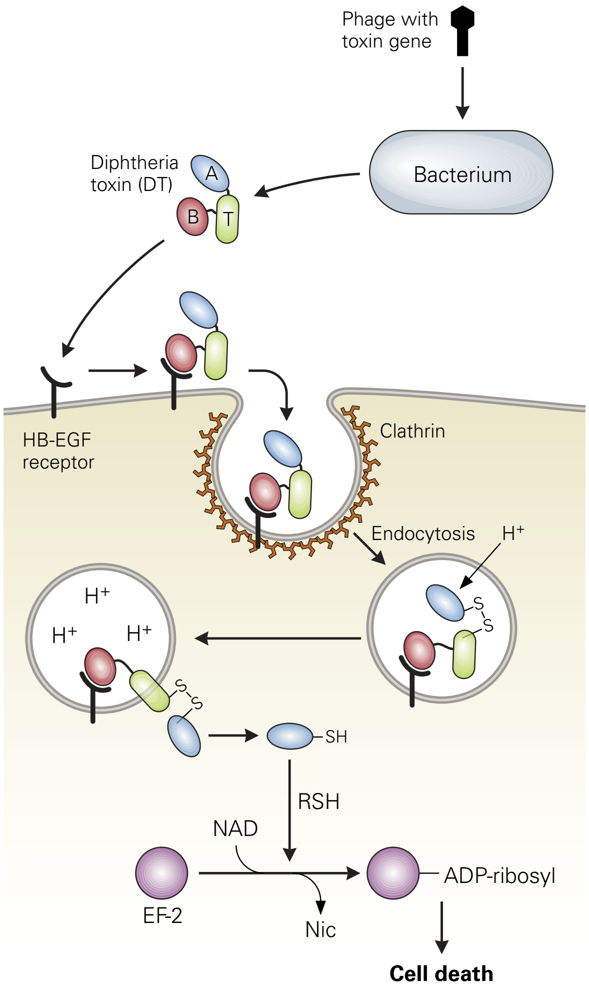


Figure 12-14. Steps in the action of diphtheria toxin. The receptor-binding domain (B domain) of diphtheria toxin (DT) binds to heparin-binding epidermal growth factor-like growth factor (HB-EGF) precursor protein, along with a putative DT receptor accessory protein 2 (DTRAP2), on the host cell surface. The membrane-bound toxin undergoes receptor-mediated endocytosis via clathrin-coated pits. The catalytic A domain is cleaved by a host protease furin, but remains attached to the rest of the toxin by a disulfide bond. Acidification of the endosome neutralizes the charges of aspartate and glutamate residues located in the loop of a helix-loop-helix region, which forms a hairpin-like structure within the T domain. Acidification also causes conformational changes in the T domain, as well as in the A and B domains. The helix-loop-helix inserts through the membrane, where the pH 7.2–7.4 of the cytosol once again deprotonates the aspartate and glutamate residues in the loop and drives the rest of the T domain to insert in the membrane. The conformational changes also bring the A domain through to the cytoplasmic side of the membrane, where the disulfide bond is reduced by thioredoxin reductase. The COPI coatomer complex, a cellular protein complex that coats vesicles transporting proteins between the Golgi apparatus and the rough ER, and heat-shock chaperone protein HSP90 also facilitate the translocation and refolding of the A domain, respectively. The A domain then catalyzes the ADP-ribosylation of the diphthamide residue of its cytoplasmic target substrate elongation factor 2 (EF-2), thereby blocking protein synthesis and causing cell death.

regions, together with protonated acidic residues in the loops of the T domain, allow the helix-loop-helix regions of the T domain to insert through the membrane. Upon exposure to the higher pH of the cytosol (pH 7.2–7.4), the aspartate and glutamate groups in the loops lose their protons, becoming once again charged. These pH-induced conformational changes along with help of the cell's coat-protein (COPI) coatomer complex is believed to drive the process of translocating the A domain through to the cytoplasmic side of the membrane. Reduction of the disulfide bond then releases the A domain into the cytosol. In line with its structural similarity to pore-forming toxins, the T domain remains behind as a pore structure in the endosomal membrane.

Once in the cytosol, the DT A domain refolds with the assistance of heat-shock protein chaperone HSP90 and then proceeds to catalyze the ADP-ribosylation of elongation factor-2 (EF-2), an essential factor in the protein synthesis machinery of eukaryotic cells (see Figure 12-12B). EF-2 participates in the elongation step of protein translation. Covalent attachment of an ADP-ribosyl group (from the cosubstrate NAD) to EF-2 renders EF-2 inactive. A single molecule of the catalytic A fragment can ADP-ribosylate enough copies of EF-2 to halt protein synthesis completely and irreversibly. Ultimately, this causes the death of the cell. Attachment of the ADP-ribosyl group occurs at an unusual derivative of histidine, called diphthamide: 2-(3-carboxyamido)-3-(trimethylamino)propyl histidine. The elaborate and unique posttranslational modification of this particular histidine residue (His-715 in mammals) to form diphthamide in EF-2 occurs in all eukaryotic cells and involves multiple biosynthetic steps (seven enzymes, Dph1–Dph7). While bacteria lack an analogous modification, *Archaea* have a deamidated version of this unusual residue, called **diphthine**. The role of diphthamide (and diphthine) is not known, but the existence of this unusual form of histidine in EF-2 in all eukaryotes and archaea suggests an important function, presumably in maintaining translation fidelity and possibly also in organismal development.

Despite this universality of action, different mammalian cell lines differ considerably in their susceptibility to killing by exogenously added DT. Differences in susceptibility of different cell lines are explained primarily by differences in number of toxin receptors expressed on the cell surface. These differences also suggest why the disease has its most drastic effects on heart and nerve cells, whose surfaces contain high concentrations of the HB-EGF precursor molecule that can be bound by the B fragment. Preferential attack of these tissues by the toxin produces heart failure and neurological symptoms (e.g., difficulty in swallowing), which are symptoms of the severe form of the diphtheria disease. Not all mammalian cells are susceptible to DT. Despite having EF-2 with diphthamide, rats are remarkably resistant to DT. This is due to the fact that rats lack the toxin receptor.

Clostridial Neurotoxins

BoNTs are produced primarily by strains of the Gram-positive spore-forming bacterium *C. botulinum*. There are seven serologically and antigenically distinct types of BoNT, serotypes A through G (see Table 12-1), so designated because antibodies raised against one serotype can neutralize that serotype, but cannot neutralize any of the other serotypes. Not all strains of *C. botulinum* produce botulinum toxin. This is explained by the finding that the toxin gene is often carried on a temperate bacteriophage. Thus, only strains lysogenized by the phage are toxin producers. Production of other BoNT serotypes, such as BoNT serotype G, are plasmid-encoded. Several other species of clostridia (*Clostridium butyricum, Clostridium argentinense,* and *Clostridium baratii*) and a couple of other Gram-positive bacteria (*Enterococcus faecium, Weissella oryzae*) can also produce BoNTs. BoNT serotypes A and B, and sometimes E and F, are most often involved in natural forms of human botulism. Serotype C is a source of avian botulism, serotype E is commonly associated with fish and aquatic sources, and serotypes B and D are primary sources of equine botulism. BoNT serotype A is considered the most potent protein toxin known for humans, with an intravenous lethal dose estimated to be 1–5 ng/kg and a half-life of several months.

Botulism. While botulism can occur as a result of an infection, it most often occurs as the result of intoxication with BoNT, which is produced by vegetative, anaerobically growing bacteria. *C. botulinum* normally grows in anaerobic soil or in lake sediments. Spores of *C. botulinum* are widely distributed in all parts of the world and have been found in soil samples taken from all parts of the United States. Spores are also found on plants growing in heavily contaminated soil. Since bees moving from plant to plant accumulate spores along with pollen, honey frequently contains *C. botulinum* spores. The concentration of spores in honey is not high (usually fewer than 7 spores per 25 g honey), but they can be a source of problems for infants (see following sections). Contamination of human foods by *C. botulinum* spores and consequent ingestion of the spores is a common occurrence, but simple ingestion of spores is generally not sufficient to cause botulism. Germination of ingested spores may occur, especially in the colon, but *C. botulinum* is usually unable to compete with the resident microbiota of the colon and thus it does not achieve high enough concentrations in the colon to cause disease.

Human botulism most often occurs when spores have the chance to germinate in foods, where the bacteria can grow and produce BoNT. Fortunately, *C. botulinum* does not grow readily in most foods since it is an obligate anaerobe and most foods contain enough dissolved oxygen to discourage germination of the spores and growth of the bacteria. Growth can, however, occur in home-canned foods that have been heated, then cooled and stored for long periods at room temperature. Heating reduces the solubility of oxygen, and most of the dissolved oxygen is lost. When the food is cooled, some oxygen redissolves, but lower regions away from the surface can remain anaerobic enough to support the germination and growth of *C. botulinum*.

Many cases of botulism have been associated with consumption of home-canned foods. Temperatures achievable by normal boiling are not always sufficiently high enough to kill the bacterial spores, which is why pressure cookers are generally preferred to sterilize canned foods. In home canning, however, the temperature in the pressure cooker sometimes still does not reach the level required to kill all of the spores. Since canned foods are often prepared in jars that are filled to the top and sealed before they are cooled, the environment in the sealed jars can be quite anaerobic, allowing for surviving spores to germinate in the cooled canned food. That said, even if BoNT is produced in a jar of canned food, botulism can still be avoided by simply boiling the canned food for 10–15 minutes before eating. Boiling does not eliminate all bacterial spores, but it is sufficient to denature and inactivate the protein toxin. Thus, most cases of food botulism are associated with consumption of contaminated canned food without adequate prior heating.

After ingestion, BoNT is absorbed from the stomach and enters the bloodstream. It is specific for neurons and attacks peripheral nerve endings at the neuromuscular junction and autonomic synapses. Inside the neuron, the toxin blocks neurotransmitter release. Thus, nerve impulses cannot be transmitted and muscles connected to the nerves are not stimulated. The result is a generalized flaccid paralysis, where the body goes limp. Symptoms begin to appear in 4 to 36 h after ingestion of contaminated food. The rapidity with which symptoms appear and the severity of the symptoms is directly proportional to the amount of toxin ingested. Initial symptoms include nausea and vomiting, as well as headache, double vision, slurred speech, and other neurological symptoms. Death occurs if the general flaccid paralysis is severe enough to interfere with breathing function, but nowadays death seldom occurs due to medical intervention such as the use of mechanical ventilators and administration of antitoxin.

Currently, there is no effective antidote available for preventing or reversing paralysis once exposure to BoNT has occurred and symptoms have initiated. Once BoNT has bound to the surface of a neuron and entered it, external intervention with antitoxin becomes ineffective. Prompt administration of neutralizing antitoxin can only reduce the severity of disease and slow its progression. The good news is that the BoNT does not kill the nerve cell, and so nerve regeneration will occur if the affected person survives the initial onslaught of toxin-mediated paralysis. Unfortunately, recovery from botulism is quite prolonged, and the time required to restore neuromuscular function depends on the type of nerve terminal affected and the serotype of BoNT involved. Recovery of the mammalian neuromuscular junction after BoNT serotype

A intoxication typically requires 4–6 months, but can take much longer (>1 year) for the human autonomic nervous system.

Although most cases of botulism are due to ingestion of preformed toxin (**food botulism**), two less common forms of botulism involve transient colonization of the body by the bacteria, followed by toxin production: **infant botulism** and **wound botulism** (Figure 12-15). Infants less than 1 year old have not yet developed a complete colonic microbiota. Since the colon provides an anaerobic environment, *C. botulinum* can sometimes colonize the infant colon and cause infant botulism. The symptoms of infant botulism are essentially the same as those of foodborne botulism, except that nausea and vomiting are not seen. Instead, constipation, which is not usually a symptom of adult botulism, is common. The fecal microbiota content of infants with botulism can reach relatively high numbers of *C. botulinum*, exceeding 3% of the total microbial content. This is presumably due to *in vivo* spore germination and bacterial outgrowth due to the slow progression of fecal contents, which is much slower in infants. Infant botulism has a slower onset than foodborne botulism with an incubation period of 3 to 30 days from the time of first exposure, but death can still occur if not treated. Because treatment with antitoxin derived from horse serum can cause hypersensitivity, special human-derived antitoxin, called BabyBIG (botulism immunoglobulin), is available for infant botulism caused by BoNT/A or BoNT/B through the California Department of Health. The financial cost for obtaining this antitoxin is quite high (tens of thousands of dollars per treatment), but with supportive care, treatment with BabyBIG can cut a hospital stay in half and the time needed for physical therapy by much more.

A deep wound can become anaerobic because tissue destruction cuts off the blood supply to the area and residual oxygen is rapidly depleted by the surrounding body cells. The environment of a deep wound can thus become anaerobic enough to support the growth of *C. botulinum* and other anaerobic opportunistic bacterial pathogens. BoNT leaks into the bloodstream from the wound area and causes wound botulism, which has symptoms similar to those of food botulism. Wound botulism is normally only seen during wartime, but civilian cases have occasionally been reported in people with severe, deep wounds that were heavily contaminated with soil. Wound botulism should be differentiated from **tetanus**, a neurotoxin-associated disease caused by another *Clostridium* species, *C. tetani* (discussed more later).

Recently, a new form of botulism has emerged: **cosmetic botulism.** BoNT/A (commercially sold as Botox) and BoNT/B (commercially sold as Myobloc) are now in widespread use for therapeutic treatment of neuromuscular disorders and for cosmetic applications (Box 12-2). Correspondingly, botulism due to inadvertent overdosing of BoNT during injections for medical or cosmetic treatments has shown a marked increase in occurrence. See Box 12-3 for a twisted tale of the perils of searching for Botox beauty at the hands of a greedy boyfriend.

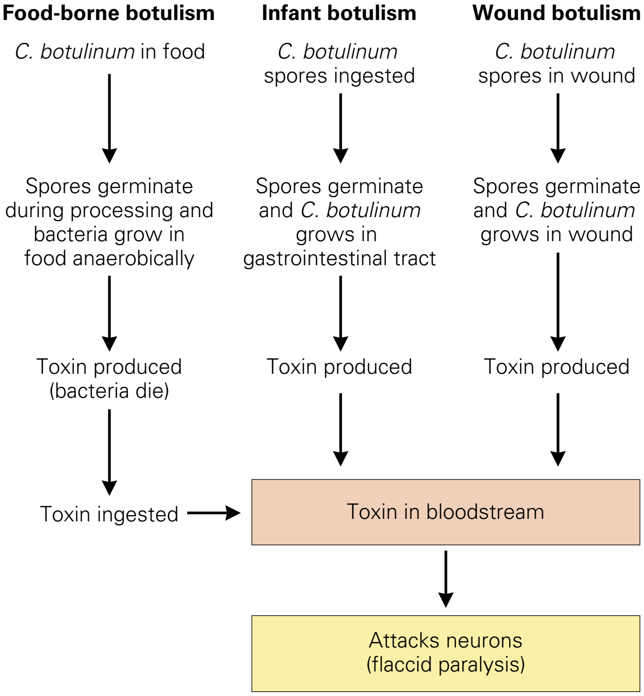

Food-borne botulism
Infant botulism
Wound botulism
C. botulinum in food
C. botulinum spores ingested
C. botulinum spores in wound
Spores germinate during processing and bacteria grow in food anaerobically
Spores germinate and C. botulinum grows in gastrointestinal tract
Spores germinate and C. botulinum grows in wound
Toxin produced (bacteria die)
Toxin produced
Toxin produced
Toxin ingested
Toxin in bloodstream
Attacks neurons (flaccid paralysis)

Figure 12-15. Comparison of transmission routes of foodborne, infant, and wound botulism.

Botulinum Neurotoxin. BoNT is synthesized and secreted from the bacteria as a single 150-kDa protein, which gets cleaved into two protein fragments, heavy chain (100 kDa) and light chain (50 kDa), which are connected by a disulfide bridge (Figure 12-8B). BoNT is actually produced by *C. botulinum* as part of an even larger complex containing other proteins beside the toxin (Figure 12-16). The complex is called **progenitor toxin** and the toxin itself is called **derivative toxin.** Derivative toxin is less effective than progenitor toxin when given orally, but is as active as progenitor toxin when injected. This has led to the conclusion that the nontoxic components of progenitor toxin help protect the derivative toxin from stomach acid and proteases through the oral route of transmission. They also help the toxin bind to and transit the stomach and/or intestinal mucosal surface.

The specificity of BoNT for peripheral neurons arises both from the specificity of toxin binding due to ganglioside and protein receptors found only on neuronal cells and from the neuron-specific action of the toxin. The bound toxin is internalized and its action inhibits release of **acetylcholine (ACh)**, a neurotransmitter involved in initiating an action potential for muscle movement at the neuromuscular junction (Figure 12-17A). The steps of intoxication and mode of action of BoNTs that lead to paralysis are illustrated in Figure 12-17B**.** After binding to its receptors through the carboxy terminal region of the heavy chain, the toxin is taken up by endocytosis into neuronal cells. Following acidification of endosomes containing the toxin, the amino terminal region of the heavy chain mediates translocation of the catalytic light chain (i.e., the A domain) to enter the cytosol of the cell. BoNT light chains (LCs) are zinc-dependent endoproteases whose intracellular targets are neuron-specific **SNARE proteins** involved in synaptic vesicle fusion that releases the ACh neurotransmitter. BoNT serotypes B, D, F, and G, as well as the related TeNT (discussed further in the next section) cleave **synaptobrevin** (also called **VAMP**), a membrane protein found in small synaptic vesicles. BoNT serotypes A and E cleave **SNAP25** and BoNT serotype C cleaves **syntaxin**. SNAP25 and syntaxin are proteins associated with the plasma membrane that form a helix bundle complex with synaptobrevin and thereby facilitate vesicle-plasma membrane fusion. The net effect of SNARE protein cleavage is that vesicle fusion does not occur, so that ACh neurotransmitter is not released to synapses, causing muscles to stop contracting and flaccid paralysis to set in.

Tetanus. Tetanus is a neurotoxin-mediated disease caused by another clostridial species, *C. tetani*. Tetanus is also known as "lockjaw" because people with classical tetanus suffer muscle spasms that can lock the jaws together. In one sense, tetanus is the opposite of botulism. In botulism, the patient suffers a flaccid paralysis, whereas in tetanus the patient develops a spastic paralysis, in which the muscles contract and do not relax, leading to stiffening of many muscles in the body and ultimately death. Tetanus starts when the bacteria colonize a deep puncture wound that becomes anoxic due to tissue damage in the area. Like *C. botulinum*, *C. tetani* is an obligate anaerobe that can grow under these conditions and produce TeNT, which diffuses away from the wound site and enters the bloodstream. TeNT acts on neurons that control the neural

Box 12-2.

Bacterial Toxins: Double-Edged Swords that Turn the Bad Guy into the Good Guy

The term "toxin" has very negative connotations. Yet scientists have managed to find beneficial uses for bacterial toxins. Since toxins often manipulate eukaryotic cell pathways that are important for reacting to external stimuli or for intracellular communication, eukaryotic cell biologists have found certain toxins to be very useful for elucidating regulatory pathways. By poisoning certain pathways, toxins allow biologists to interrupt these pathways biochemically and thereby study the importance of particular steps in the pathway for cellular functions. Studies of this type have provided much information about the functioning of eukaryotic cells, information that has already proven useful in a variety of medical areas.

Botulinum neurotoxin has emerged as one of the white knights of toxin-based therapies. For many years now, botulinum neurotoxin (sold as Botox) has been used as an FDA-approved drug to treat painful, disabling muscle spasms of various types (dystonias). Examples of such dystonias are strabismus (crossed eyes) and painful spasms of the face and neck. Dystonias were once thought to be psychosomatic in origin, but attempts to treat them using various forms of psychotherapy were largely unsuccessful. Their cause is still unknown. Until the advent of Botox therapy, the only therapy that sometimes worked was the expensive and potentially dangerous surgical destruction of the nerve endings in the area affected by the spasms. Botox therapy is generally acknowledged to be far superior to surgical destruction of nerve endings and thus represents the first successful therapy of dystonias. Since botulinum neurotoxin prevents transmission of nerve impulses, small injections of toxin in the affected area counter the spasms and give temporary relief. The injections must be repeated at intervals, because recovery at the nerve endings does occur.

Botox has also found favor with cosmetic surgeons who are using it to reduce the depth of wrinkles. Indeed, the cosmetic use of Botox has soared over the past 15 years and has now become a multi-billion-dollar industry. More recently, Botox has found many other biomedical applications. Botox has been administered to cerebral palsy patients in an effort to help them control the movement of their limbs. It has been used to treat migraines, excessive sweating, urinary incontinence, and hypersalivation (drooling). One remarkably successful use for Botox is in the treatment of facial wounds to prevent severe scarring during the wound healing process.

Sources:

Carruthers A, Kane MA, Flynn TC, Huang P, Kim SD, Solish N, Kaeuper G. 2013. The convergence of medicine and neurotoxins: a focus on botulinum toxin type A and its application in aesthetic medicine—a global, evidence-based botulinum toxin consensus education initiative: part I: botulinum toxin in clinical and cosmetic practice. *Dermatol Surg* **39**(3pt2)**:**493–509.

Ziade M, Domergue S, Batifol D, Jreige R, Sebbane M, Goudot P, Yachouh J. 2013. Use of botulinum toxin type A to improve treatment of facial wounds: a prospective randomised study. *J Plast Reconstr Aesthet Surg* **66:**209–214.

Box 12-3.
A Lesson in Why Not to Trust a Greedy Beau with Your Beauty

The Botox craze has intoxicated (pun intended) many a wrinkled glamour-seeker desirous of a youthful glow. No longer restricted to the Hollywood stars, Botox injections are a new and booming market. However, the demand for this rejuvenating cosmetic has spurred some dubious activities as well. In the United States, Botox is manufactured and sold only by firms licensed by the FDA to do so. Botox is normally sold in 100-unit (5-dose) vials at a cost of $1,000–2,000 per vial, where one treatment lasts for 4–6 months. The problem is that some individuals do not want to pay that much for a cosmetic, which has led to sales of non-FDA approved Botox knockoffs. Consequently, there has been a rise in the number of cases of misuses of the toxin.

One such notorious case occurred in 2004, when an osteopathic physician, Bach McComb, working at a health salon in Florida injected his coworker and girlfriend, Alma Jane Hall, and another couple, Eric and Bonnie Kaplan, at the salon with a non-FDA approved Botox knockoff. The injections ended up being several thousand times over the dose normally administered for antiwrinkle treatment. Shortly after the treatments, Hall and McComb traveled to New Jersey to visit his family for the Thanksgiving holiday. Hall had started to show symptoms of botulism on the plane trip, but McComb refused to get her help and instead attempted to treat her with ice packs and then an ice bath at his mother's place, before a family member finally called 911. Once it was determined that she indeed had botulism, Hall received antitoxin and was transferred to the University of Medicine and Dentistry of New Jersey (UMDNJ) under the care of Dr. Steven Marcus, Professor of Preventive Medicine and Community Health and executive director of the New Jersey Poison Information and Education System (i.e., the NJ Poison Control Center). Hall's ordeal and recovery period lasted more than two years, all of which was thoroughly documented (with Hall's consent) and has provided enormously valuable information for medical practitioners and biomedical researchers. Back in Florida, the other two victims fell seriously ill that same weekend and were also hospitalized, where they received antitoxin and supportive care. The boyfriend, who claimed to have also injected himself and was also hospitalized in New Jersey, received three years in prison for "causing reckless harm to others." Oh, and he is no longer Hall's boyfriend.

Sources:

Kuczynski A. 2004. Is it Botox, or is it bogus? *The New York Times.* http://www.nytimes.com/2004/12/05/fashion/05BOTU.html

Barbano R, Karim H, Kamin SS, McArdle J, Marcus S. 2006. Risks of erasing wrinkles: buyer beware! *Neurology* **67:**E17–E18.

feedback that tells flexed muscles to relax after having performed a task. In effect, it prevents these neurons from signaling the relaxation after a muscle contraction. Hence the spastic paralysis that characterizes tetanus.

Tetanus was never a very common disease in the developed world, but it was much feared because of its high fatality rate and gruesome symptoms. Victims develop painfully bowed spines, clenched arm and leg muscles, and locked jaws. Tetanus is almost invariably fatal. In developed countries today, tetanus is virtually unknown because of the tetanus vaccine (the T in the common DTaP vaccine). Worldwide, tetanus remains a major cause of infant deaths. In any listing of the top killers of infants in the developing world, infant tetanus is right up there in the top 15, perhaps not so common as HIV or diarrheal disease, but still pervasive. The custom in some parts of the world of packing the umbilical stump of a newborn with cattle dung contributes to this grim statistic. Animal dung may possibly prevent other infections, but it is also a prime source of *C. tetani*. A number of intervention programs are underway to reduce this practice.

Different Toxins, Same Mechanism. For years, botulism and tetanus were treated as completely different diseases, even though the toxins responsible for the symptoms were both neurotoxins. Thus, it came as a surprise when examination of the deduced amino acid sequences of the two toxin genes revealed that the two proteins shared a considerable amount of sequence similarity and were both zinc-dependent endopeptidases. Ultimately, this insight from sequence gazing led to the discovery that TeNT, like some of the BoNT serotypes, cleaves synaptobrevin. But, if the two toxins have exactly the same mode of action, how could these two toxins cause such different types of effects (flaccid paralysis vs. spastic paralysis)? The answer appears to lie in the different cell specificities of the binding regions of the two toxins. BoNTs target peripheral neurons. If TeNT is administered so that it only acts locally on peripheral nerves, it too causes flaccid paralysis. In high enough quantities and especially if it enters the circulation, however, TeNT acts on the central nervous system and causes spastic paralysis. TeNT also targets different neuronal receptors from that of the BoNTs. Although TeNT and BoNTs bind to the neuromuscular junction, their in-

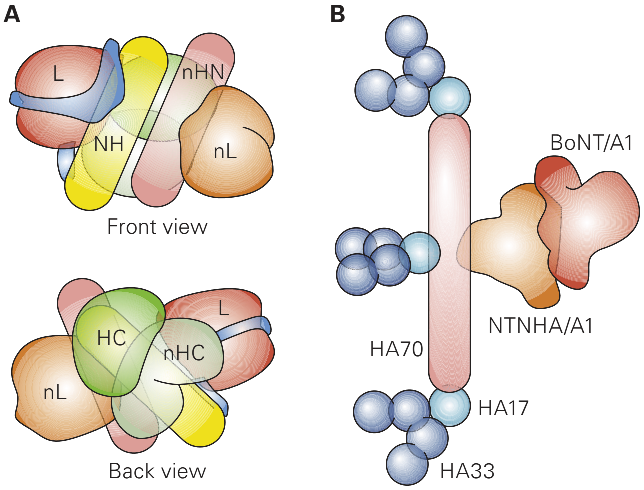


Figure 12-16. Structure of the BoNT/A1-NTNH/A1 heterodimer and the progenitor toxin complex. (A) BoNT/A1 is produced by *C. botulinum* as a heterodimeric complex with the nontoxic nonhemaglutinin (NTNH/A1), a protein that is remarkably similar in structure to BoNT/A1 but lacks the zinc-binding motif of the active site of the metalloprotease domain and so is inactive. Shown are front and back views of the complex. **(B)** The BoNT/A1-NTNH/A1 complex is docked with a large progenitor complex, comprised of six HA33 proteins, three HA17 proteins, and three HA70 proteins. While NTNH/A1 serves to protect the toxin from harsh extracellular environments, including that of the stomach and gut, the progenitor complex serves to deliver the BoNT/A1-NTNH/A1 complex across the intestinal epithelium and into the body's circulation system. Consistent with this, the HA proteins have multiple sugar-binding sites that are postulated to bind to the intestinal epithelial cells. The point during intoxication when the complex dissociates to release BoNT/A1 is not yet known, but it has been proposed that the slightly basic conditions of the basal lamina and in intramuscular fluids might allow for dissociation.

tracellular actions take place at different levels of the nervous system. Unlike BoNTs, TeNT undergoes retrograde transport to the cell body of spinal cord motor neurons and is there translocated and cleaves synaptobrevin at inhibitory synapses. This differential trafficking within the neuron has been interpreted to be a consequence of binding to different surface receptors, which trigger sorting to different locations in the cell.

The discovery of the target of these two toxins illustrates a common theme in toxin research—bacterial toxins can become powerful tools for the study of mammalian cell function. The finding that TeNT and BoNTs cleaved different neuron-specific SNARE proteins excited researchers interested in neurobiology because the function of the SNARE proteins had previously been unknown. Thus, TeNT and BoNTs provided a new reagent that helped researchers understand how these proteins function.

Cholera Toxin

The multi-subunit AB_5 CT (see Figure 12-8C) is secreted by Gram-negative *V. cholerae* that harbor the prophage CTXϕ. Genetic evidence suggests that the *ctxAB* gene cluster encoding CT is located on this prophage, which is transmitted through HGT (see chapter 7) among *V. cholerae* and other environmental *Vibrio* species, such as *Vibrio mimicus*, by filamentous vibriophage CTXϕ (Figure 12-18). Furthermore, it appears that the *tcp* genes encoding the toxin-coregulated pilus (TCP), which serve as receptors for the CTXϕ phage, are located on a second prophage, VPIϕ. It is posited that frequent HGT events among *Vibrio* in the environment have contributed to the emergence of new epidemic strains of toxin-producing *V. cholerae*.

Vibrio cholerae. *V. cholerae* are primarily found associated with zooplankton, phytoplankton, or marine snow (debris particles) in marine environments, especially brackish water. Seasonal outbreaks of cholera in endemic regions of the world are strongly correlated with abundances (blooms) of zooplankton or phytoplankton, to which historical O1 serotype strains of the El Tor biotype attach via a type 4 pilus, called mannose-sensitive hemagglutinin (MSHA) (Figure 12-19). See Box 12-4 for a clever way that one group of researchers exploited this knowledge about how *Vibrio* adheres to marine snow to devise a highly cost-effective method to reduce cholera outbreaks in poor regions where cholera is endemic.

When ingested through contaminated water, the *V. cholerae* uses flagellar motility (see Figure 11-3) to penetrate through the viscous mucus layer and attach to epithelial cells in the small intestine via several surface adhesins, including another type 4 pilus, the TCP pilus mentioned previously, which promotes formation of microcolonies on the surface and production of CT (among other toxins) into the lumen of the gut. The bacteria are noninvasive, so little or no inflammatory response results. However, CT triggers a massive diarrheal response, such that a person can lose up to 20 liters of fluid per day (Figure 12-20A). The stool generated by cholera diarrhea has a very characteristic appearance as pale milky water with flecks of mucus, resembling water that has been used to wash rice (hence the name **rice-water stool**). Without rehydration therapy (see the following section), death can result from dehydration.

Mechanism of Cholera Toxin-Mediated Diarrhea. The B subunits of CT form a pentameric complex that binds to GM1 gangliosides on intestinal epithelial cells (see Figure 12-8C). After internalization via receptor-mediated endocytosis into recycling endosomes, the toxin is processed by furin and transported through retrograde trafficking to the Golgi apparatus and then the ER, similar to that described

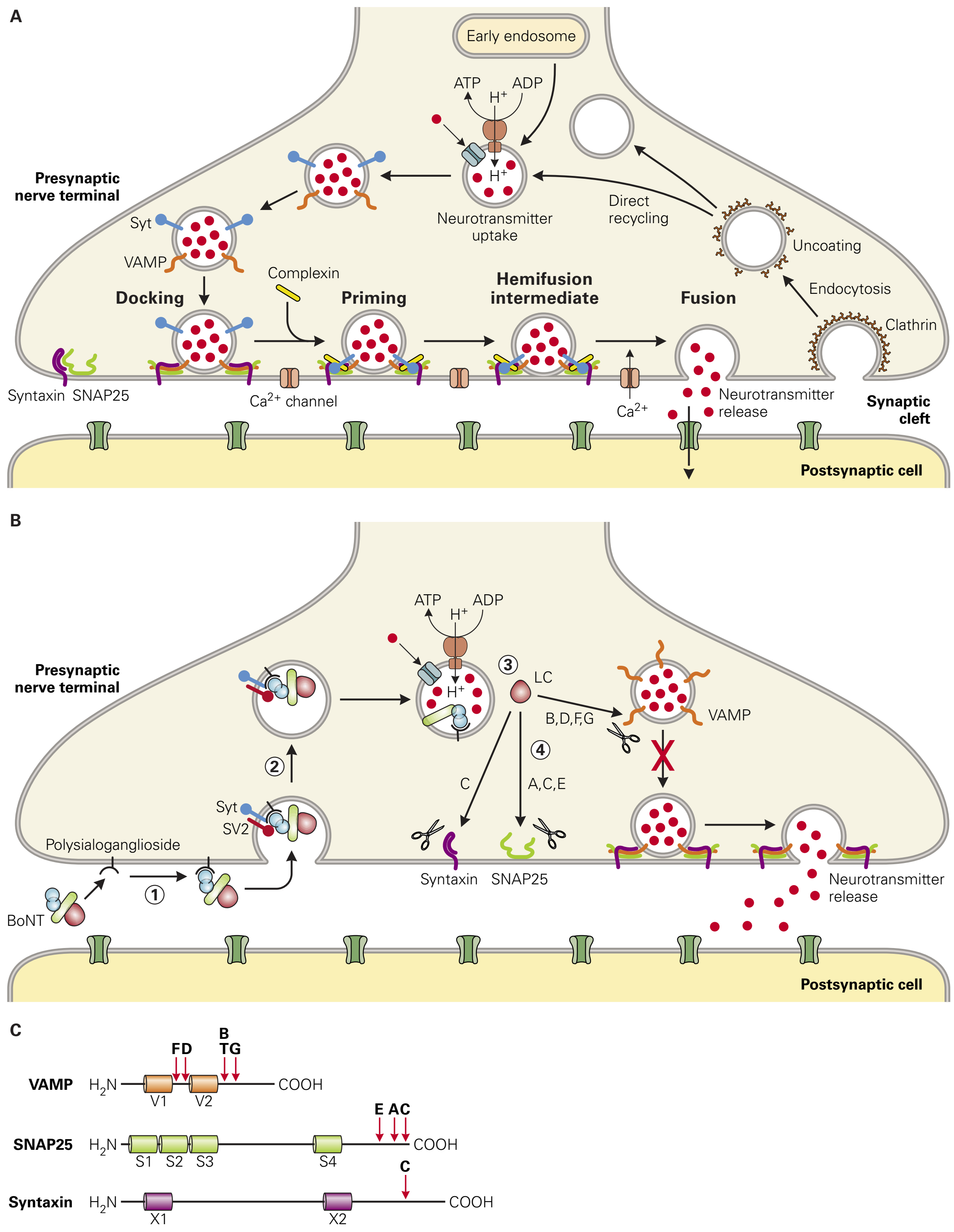

A
Early endosome
ATP
ADP
H^+
H^+
Presynaptic nerve terminal
Neurotransmitter uptake
Direct recycling
Syt
VAMP
Uncoating
Endocytosis
Complexin
Docking
Priming
Hemifusion intermediate
Fusion
Clathrin
Syntaxin
SNAP25
Ca^{2+} channel
Ca^{2+}
Neurotransmitter release
Synaptic cleft
Postsynaptic cell
B
ATP
ADP
H^+
H^+
Presynaptic nerve terminal
③
LC
B,D,F,G
VAMP
②
④
C
A,C,E
Syt
SV2
Polysialoganglioside
BoNT
①
Syntaxin
SNAP25
Neurotransmitter release
Postsynaptic cell
C
FD
B
TG
VAMP
H_2N
COOH
V1
V2
E AC
SNAP25
H_2N
COOH
S1
S2
S3
S4
C
Syntaxin
H_2N
COOH
X1
X2

Figure 12-17. Action of botulinum neurotoxins at the motor neuron synapse. (A) Normal neurotransmission at the synapse of neuromuscular junctions. Neurotransmitters such as acetylcholine (ACh), synthesized in the cytosol, are transported and stored in presynaptic vesicles at the nerve terminal. Two membrane proteins in the presynaptic vesicle, VAMP (also known as synaptobrevin) and synaptotagmin (Syt), interact with a complex of Syntaxin, SNAP25, and Munc18 on the presynaptic plasma membrane to mediate vesicle docking and formation of the primed state. Membrane fusion is blocked by binding to the protein complexin until depolarization of the nerve terminal membrane, which triggers Ca^{2+} influx and large conformational changes that initiate membrane fusion and release of neurotransmitter into the synaptic cleft. The neurotransmitters bind to postsynaptic receptors on the muscle, which in turn initiates an action potential and muscle movement response. **(B)** The action of BoNTs occurs in four steps: (**1**) binding of the C-terminal portion of the heavy chain (H_C) to presynaptic membrane receptors; (**2**) uptake of the toxin into a synaptic endocytic vesicle; (**3**) translocation of the catalytic light chain (LC) into the cytosol via the N-terminal portion of the heavy chain (H_N); and (**4**) BoNT-LC-mediated proteolytic cleavage of neuron-specific SNARE proteins involved in synaptic vesicle fusion with the plasma membrane, which prevents membrane fusion and release of the neurotransmitter, acetylcholine (ACh). **(C)** LCs from different BoNT serotypes target different SNARE proteins.

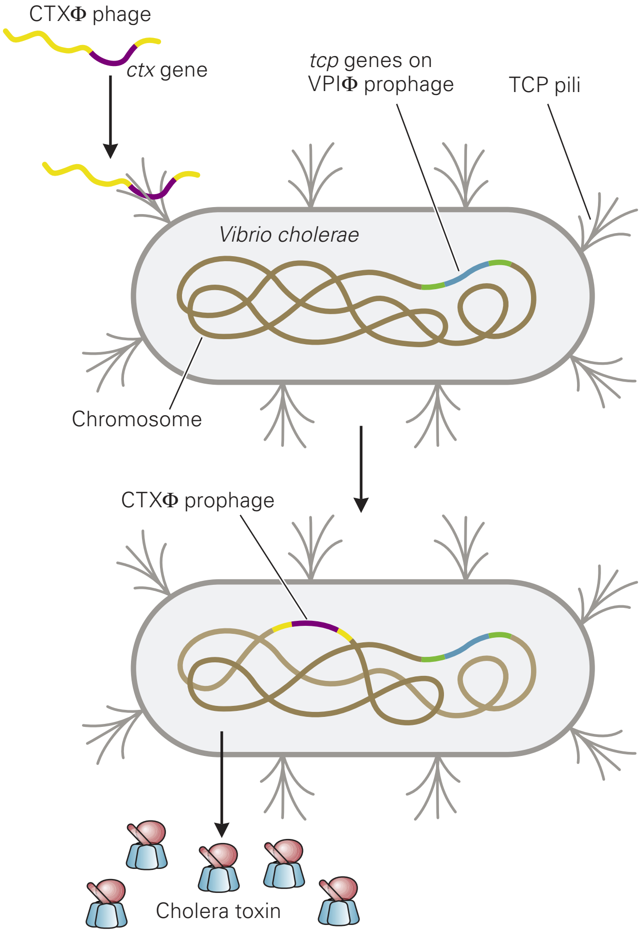


Figure 12-18. Only *Vibrio cholerae* strains expressing toxin-coregulated pili (TCP) can acquire the CT-encoding filamentous CTXϕ phage. Non-toxin producing *V. cholerae* strains harboring the prophage VPIϕ express TCP pili on their surfaces and can be infected with CTXϕ phage that carry the *ctxBA* gene cluster. The resulting bacterial strains can then produce CT and cause cholera disease.

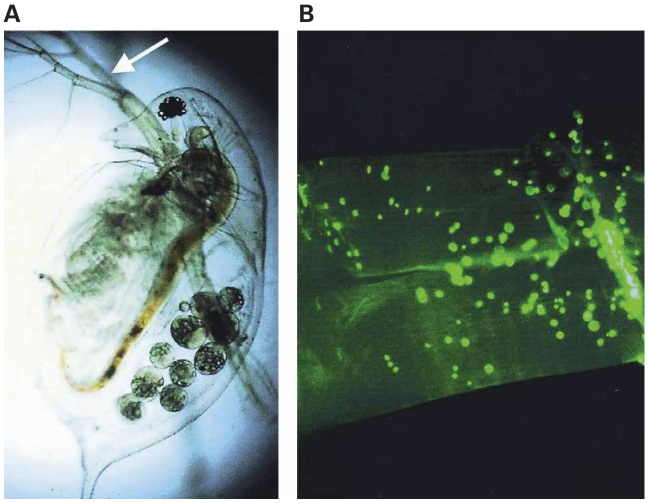


Figure 12-19. *Vibrio cholerae* attach to zooplankton and marine snow. Epidemic strains of *Vibrio cholerae* O1 El Tor biotype and *V. cholerae* O139 strains are found in marine environments attached as biofilms to zooplankton or marine snow (tiny particles of sand, seashells, or other debris). Adherence in O1 El Tor and O139 strains, but not classical O1 strains, is mediated through mannose-sensitive hemagglutinin (MSHA) pili. **(A)** Light micrograph of zooplankton, *Daphnia pulex*. **(B)** Fluorescence micrograph of GFP-expressing *V. cholerae* O1 El Tor biotype bacteria located on the surface of *D. pulex* at the arrow in the light micrograph of panel (A). Bright green dots are individual bacteria. Bacteria also colonize into joint crevices on the exoskeletons (long, bright green patch). Reprinted from Chiavelli DA, Marsh JW, Taylor RK. 2001. *Appl Environ Microbiol* **67:**3220–3225, with permission.

for STx (see Figure 12-11). Once in the ER, the A1 subunit is dissociated from the $A2B_5$ complex with the assistance of protein disulfide isomerase (PDI) and ER oxidoreductin (Ero1), and translocated via the Sec61p complex into the cytosol, where it avoids ubiquitination and proteasome degradation and refolds.

Once the A1 subunit is delivered to the epithelial cell cytosol, its ADP-ribosyltransferase activity covalently modifies the GTPase α-subunit of the stimulatory heterotrimeric Gs protein that constitutively up-regulates the cell's adenylate cyclase (AC) (Figure 12-20B). The stimulated AC converts ATP into cAMP, greatly increasing cellular cAMP levels, which in turn stimulates cAMP-dependent protein kinase (PKA). PKA is a key regulator of various cellular signaling processes, including Na^+ and Cl^- ion transporters (pumps and channels) that maintain body electrolyte and water homeostasis (Figure 12-20C). CT-mediated activation of PKA leads to phosphorylation and inhibition of the Na^+/H^+ exchanger and blockade of Na^+ adsorption by villus absorptive enterocytes. In crypt cells, CT-mediated activation of PKA leads to phosphorylation and stimulation of the major Cl^- ion channel, the **cystic fibrosis transmembrane conductance regulator (CFTR)**. This causes a net efflux of Na^+ and Cl^- into the lumen of the gut (Figure 12-20D). Since water will follow the osmotic gradient, this results in rapid loss of water into the lumen, causing massive intestinal distention and diarrhea. CT-mediated water efflux into the lumen of the gut can be measured using the rabbit ileal loop assay (see Figure 2-10).

Oral Rehydration Therapy (ORT). The mainstay for preventing dehydration and death in cholera patients is **oral rehydration therapy (ORT)**. The current WHO ORT formula recommended for children consists of: 2.6 g NaCl, 1.5 g KCl, 2.9 g Na_3^+-citrate (or 2.5 g $NaHCO_3$), and 13.5 g anhydrous glucose per liter of clean water. A number of other formulations are available, including homemade recipes (6 teaspoons of sugar, 0.5 teaspoon of salt in 1 liter of water). The key aspect of ORT is the inclusion of a mixture of

Box 12-4.

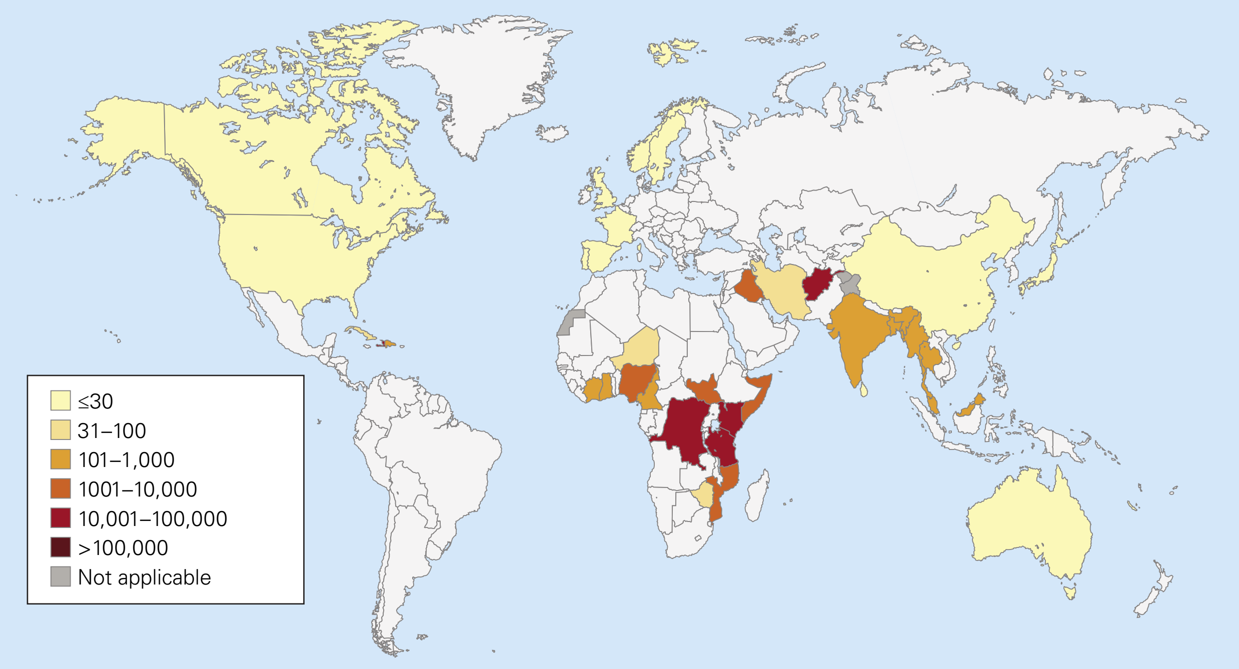

≤30
31–100
101–1,000
1001–10,000
10,001–100,000
>100,000
Not applicable

Figure 1. Global map of countries reporting cholera during 2015, based on WHO database.

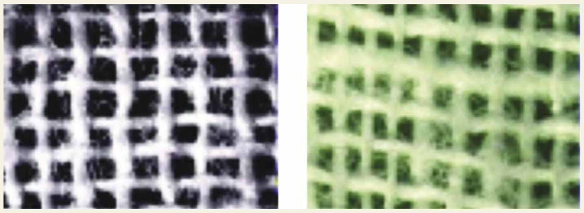
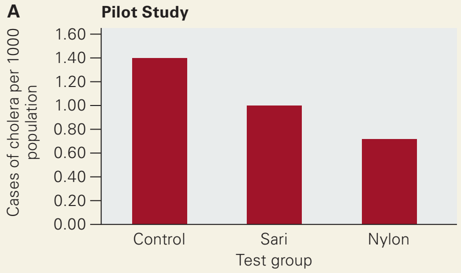

New Sari
Old Sari

Figure 2. Electron micrographs of a single layer of sari cloth used to filter water. The sari cloth has a pore size of 100–150 μm, but if folded into at least four layers, yields a pore size of about 20 μm, which is effective in retaining *Vibrio*-attached marine snow. Reproduced from Colwell RR, Huq A, Islam MS, Aziz KM, Yunus M, Khan NH, Mahmud A, Sack RB, Nair GB, Chakraborty J, Sack DA, Russek-Cohen E. 2003. *Proc Natl Acad Sci U S A* **100**:1051–1055, with permission.



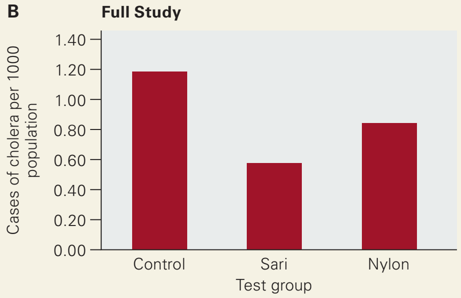


Figure 3. Cholera incidences among villages using nothing (control group), sari cloth (less expensive), or nylon membrane (more expensive) to filter water sources. Adapted from Colwell RR, Huq A, Islam MS, Aziz KM, Yunus M, Khan NH, Mahmud A, Sack RB, Nair GB, Chakraborty J, Sack DA, Russek-Cohen E. 2003. *Proc Natl Acad Sci U S A* **100**:1051–1055, with permission.

NaCl plus glucose to bypass the inhibited Na^+/H^+ exchanger and force the Na^+-glucose cotransporter to bring Na^+ into cells, thereby reversing the water flow direction caused by CT action. Since its first formulation in the 1940s, ORT became a common therapy for cholera (and other diarrheal diseases) by the 1970s and has proven to be over 90% effective in preventing dehydration and death from cholera, thereby saving millions of lives.

Toxin-Based Therapeutics and Research Tools

Toxins have been very useful research tools for cell biologists and immunologists. In terms of understanding their mechanisms of action, we now have sufficient insights into how toxins work, and we can now use them for our own benefit. In developmental biology, researchers have also used immunotoxins to knock out certain cell types by expression of the toxin under cell type-specific mammalian promoters that turn on only during certain stages of development. Elimination of that cell type can provide information about the role and importance of that cell type in development.

One example already mentioned previously and in Box 12-2 is the application of BoNT to treat neurological conditions like muscle spasms, crossed eyes, or muscle dystonia, to aid in wound healing, to reduce pain, or to reduce facial wrinkles. Because toxins are highly efficient cargo delivery vehicles, they have also been used for intracellular delivery of peptide antigens to immune cells in novel vaccine strategies (more on toxin-based epitope-delivery in chapter 17).

Not all bacterial toxins are directed at human cells. Some toxins are being used for beneficial agricultural purposes. For example, cell-killing toxins produced by the Gram-positive soil bacterium *Bacillus thuringiensis*, the so-called **Bt toxins** (**crystal** or **Cry toxins**), are widely used as insecticides in agriculture because they selectively attack a small subset of harmful insects (moths, flies, mosquitoes, beetles, ants, and nematodes), but not beneficial insects (bees and butterflies).

Immunotoxins

Another biomedical application mentioned in chapter 5 is the use of cell-killing toxins, such as the catalytic domain of

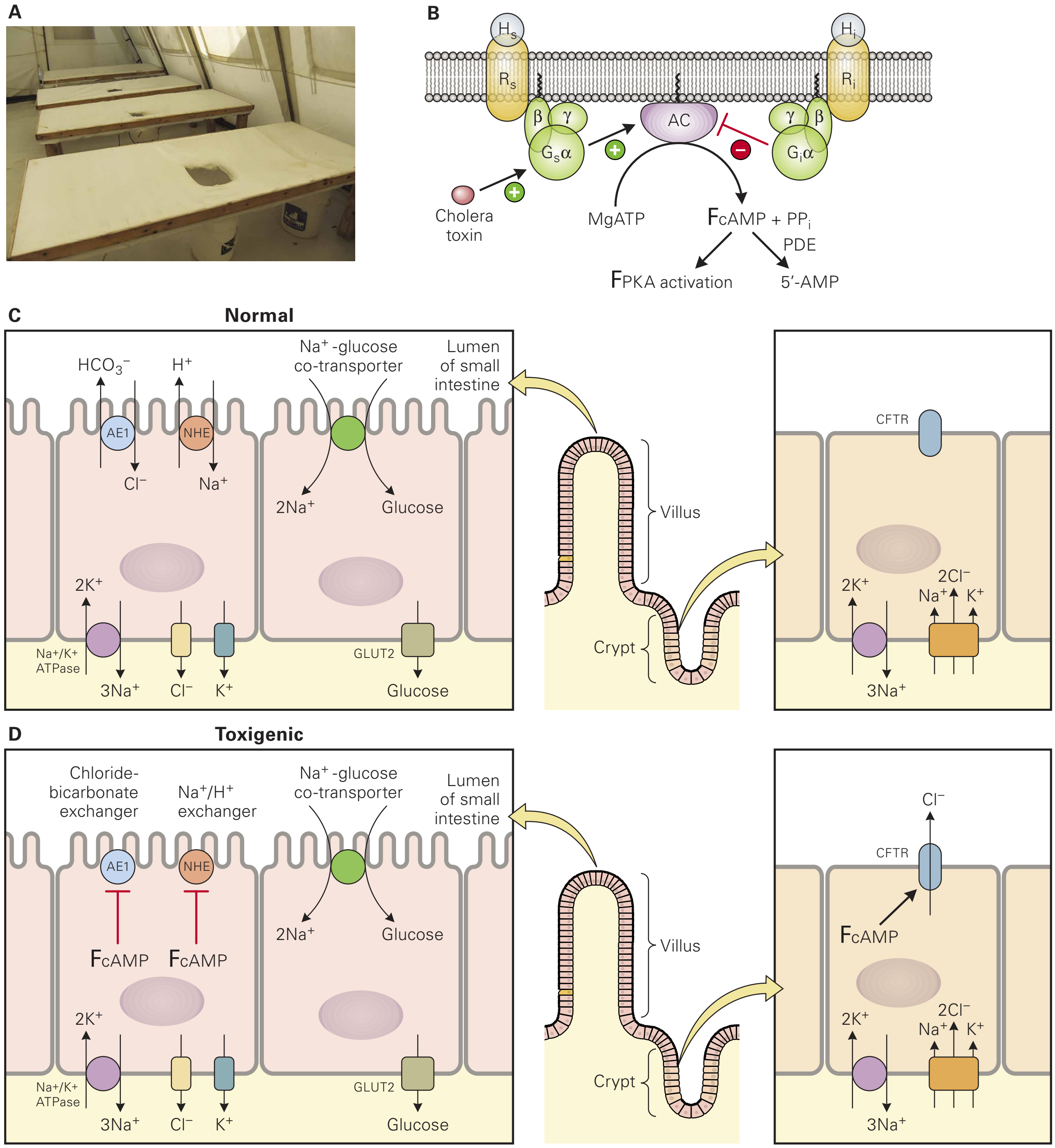

A
B
Hs
Rs
β
γ
Gsα
AC
γ
β
Hi
Ri
Giα
Cholera toxin
MgATP
FcAMP + PPi
PDE
FPKA activation
5′-AMP
C
Normal
HCO3−
H+
AE1
NHE
Cl−
Na+
Na+-glucose co-transporter
2Na+
Glucose
Lumen of small intestine
2K+
Na+/K+ ATPase
3Na+
Cl−
K+
GLUT2
Glucose
Villus
Crypt
CFTR
2K+
2Cl−
Na+
K+
3Na+
D
Toxigenic
Chloride-bicarbonate exchanger
Na+/H+ exchanger
AE1
NHE
FcAMP
FcAMP
Na+-glucose co-transporter
2Na+
Glucose
Lumen of small intestine
2K+
Na+/K+ ATPase
3Na+
Cl−
K+
GLUT2
Glucose
Villus
Crypt
Cl−
CFTR
FcAMP
2K+
2Cl−
Na+
K+
3Na+

Figure 12-20. Molecular basis of cholera toxin-mediated diarrhea and oral rehydration therapy. Cholera toxin (CT) triggers massive diarrhea, such that a person can lose up to 20 liters of fluid in a day. Without oral rehydration therapy, death can result from dehydration. **(A)** Shown is a makeshift treatment facility in Haiti for cholera patients with cholera cots (left panel) that have holes used to collect and measure fluid loss as rice-water stool (right panel) in containers underneath. Image ©2014 Melanie Blokesch, used with permission. **(B)** CT entry and mechanism of action on Gs protein. The A1 subunit catalyzes the ADP-ribosylation of the α-subunit of the stimulatory heterotrimeric Gs protein, which constitutively activates its downstream effector adenylate cyclase (AC), greatly increasing cellular cAMP levels. This leads to long-lasting stimulation of cAMP-dependent protein kinase (PKA) and downstream PKA-dependent phosphorylation of cellular proteins. PDE, cAMP phosphodiesterase; R, G-protein-coupled receptor; H, hormone. **(C)** Normal flow of electrolytes in the intestine leads to a net uptake of Na^+ ions, with water and Cl^- ions following the osmotic gradient. Different epithelial cells in the villi and crypts of the intestine carry out various transport functions. **(D)** CT action results in PKA-mediated phosphorylation that down-regulates (blocks) neutral Na^+ adsorption by the absorptive enterocytes in villi, while at the same time increasing Cl^- secretion by nearby crypt cells through activation of the cystic fibrosis transmembrane conductance regulator (CFTR), a major Cl^- ion channel in the gut. This causes a net accumulation of Na^+ and Cl^- in the lumen of the gut, which causes rapid loss of water into the lumen, resulting in massive diarrhea. Oral rehydration therapy (ORT) is the current mainstay for preventing dehydration and death in cholera patients. The mixture of NaCl + glucose in the ORT formula exploits the Na^+-glucose cotransporter to bring Na^+ into cells, thereby reversing the efflux of water caused by CT action.

DT, *P. aeruginosa* exotoxin A, or ricin, to selectively ablate or kill certain cells. This approach has been applied toward the killing of cancer cells by coupling the cell-killing portion of the toxin with selective cancer cell-targeting antibodies or ligands. These new **immunotoxin** therapeutic agents have revolutionized treatment options for prostate, breast, bladder, ovarian, colorectal, and pancreatic cancers, as well as leukemia and melanoma. Because of DT's potent cell-killing ability, it was used as the prototype for new therapeutic approaches against cancer cells or viral-infected cells. Early immunotoxin approaches used hybrid fusion proteins between the catalytic domain (the A fragment) of DT and cell-targeting, receptor-binding proteins, such as antibodies or receptor ligands that could selectively target the fusion protein to particular cancer cells or viral-infected cells. These hybrid proteins are referred to as immunotoxins because they contain a cell-killing part (the A fragment of DT) and a cell-targeting part (an antibody or receptor ligand). For example, the A fragment of DT fused with IL-2, which recognizes, enters, and kills IL-2 receptor-expressing lymphoma and leukemia cells, showed good response in phase I and II clinical trials. In other trials, a conjugate of human transferrin with the A fragment of DT showed good clinical efficacy when it was administered intratumorally or when it was infused in the brain tissue surrounding a malignant glioma (brain tumor).

From clinical trials, researchers have learned that leukemia cells in the blood are exposed to high immunotoxin concentrations. In addition, the immune system is impaired in many hematologic malignancies, such that antibody responses against the immunotoxin are also impaired. Since many cycles of immunotoxin therapy can be given without antibody formation against the therapeutic agent, immunotoxins are more effective against leukemia-like diseases. For solid tumors, immunotoxins can reach the cells within the tumors only by slow diffusion and in low concentrations. Since the immune system is still intact in patients with solid tumors, antibody formation against the DT-based immunotoxins prevents multiple treatments for these cases. The only way around this problem is by direct injection into the center of the tumor. In addition, most people are immunized against DT and have antibodies that can neutralize its activity, so use of DT is not ideal for most therapeutic applications. Consequently, researchers have turned to other cell-killing toxins as alternatives, including the ADP-ribosylating domain of *Pseudomonas* exotoxin A and **ricin** (a plant toxin with catalytic activity like STx).

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Questions

1. The B subunits of A-B toxins are being used as vaccine components without any chemical treatment. Why is it safe to use B subunits as they are?
2. What would happen if you injected the A chain of diphtheria toxin into your bloodstream? Could the A chain be used as a vaccine component?
3. How could you use diphtheria toxin A subunit to kill cancer cells, as some scientists are trying to do? What would be the advantages of such a treatment? What would be the disadvantages?
4. Why is vaccination usually the best way to prevent diseases caused by A-B toxins?
5. Could vaccination help prevent botulism, gangrene, or streptococcal cellulitis? Is vaccination a feasible solution to these problems?
6. Can you think of any way that botulinum neurotoxin or tetanus neurotoxin could aid the bacterium that produces it to live a longer and fuller life?
7. How could a toxin that has a single target inside a mammalian cell produce different symptoms? Use two of the toxins listed in Table 12-1 as examples.
8. How do A-B toxins resemble or differ from cytotoxins that affect bacterial membrane structure or superantigens?
9. What is the significance of the fact that subcytotoxic doses of membrane-active toxins might contribute to septic shock?
10. How does endotoxin differ in its mode of action from an A-B type toxin?
11. If a science writer or reporter asked you to explain briefly what a toxin is, how would you answer that question? That is, are there any unifying features of toxins that can be easily understood?
12. For the following statements, indicate whether the statement is true or false.

    Bacterial endotoxin is the lipid A moiety of LPS and acts by inhibiting the host inflammatory response.________

    A few microorganisms are pathogenic solely because of the toxins they produce. (In other words, invasion of the host tissue is not required for pathogenicity.)______

    The pertussis toxin is the cause of the characteristic paroxysmal cough seen in patients with whooping cough.______

    Cholera toxin causes diarrhea by destroying G proteins.______
13. The staphylococcal enterotoxin and toxic shock syndrome toxin are known as __________________ that can nonspecifically stimulate large populations of T cells to produce __________________.
14. Botulinum toxin is a ______________________that binds to the synapses of motor neurons, cleaves ________________, and prevents the release of the neurotransmitter, ____________________.
15. Toxins that promote nonspecific interaction of MHC II cells and T cells and cause excessive activation of T cells are known as ____________________.
16. Will heating food contaminated with botulinum neurotoxin protect you from foodborne disease? Why or why not?
17. Suppose you have two A-B type exotoxins, A-B and A'-B'. A-B binds specifically to neurons and prevents a neuron-specific function, which is essential for nerve pulse transmission. A'-B' binds specifically to kidney cells and stops protein synthesis.
    a. What would a hybrid toxin, A'-B, do to kidney cells? Provide your rationale.
    b. What would a hybrid toxin, A-B', do to kidney cells? Provide your rationale.

   c. What would a hybrid toxin, A'-B, do to neurons? Provide your rationale.
   d. What would a hybrid toxin, A-B', do to neurons? Provide your rationale.

18. Suppose you have two A-B type exotoxins, A-B and A'-B'. A-B binds specifically to all MHC-II$^+$ cells and blocks vesicle trafficking. A'-B' binds specifically to all MHC-I$^+$ cells and stops protein synthesis.
    a. What would a hybrid toxin, A'-B, do to fibroblast (skin) cells? Provide your rationale.
    b. What would a hybrid toxin, A-B', do to fibroblasts? Provide your rationale.
    c. What would a hybrid toxin, A'-B, do to macrophages? Provide your rationale.
    d. What would a hybrid toxin, A-B', do to macrophages? Provide your rationale.

19. Give a possible set of bacterial virulence factors and what properties they have that would explain each of the following observations:
    a. an ID_{50} value of 10^5, but an LD_{50} value of 10
    b. an LD_{50} value of 10^5 in mice, but an LD_{50} value of 10 in humans under similar exposure conditions
    c. development of an autoimmune response after the infection

20. In this chapter we have studied a number of toxins that are responsible for many of the disease symptoms associated with potent pathogens. For each of the following toxins, describe the mechanism of action on host cells in terms of the structure of the toxin, how it gains access to its target, what it does to its target, and the resultant outcome.
    a. Diphtheria toxin
    b. Shiga toxin
    c. *E. coli* heat-stabile toxin
    d. Cholera toxin
    e. Superantigen
    f. YopE
    g. Listeriolysin O

21. Cholera treatment involves consumption of a mixture of the following, except
    a. sodium chloride.
    b. sodium bicarbonate.
    c. potassium chloride.
    d. glucose.
    e. magnesium chloride.

Solving Problems In Bacterial Pathogenesis

1. You are a researcher characterizing two newly discovered toxins. You have been able to generate research data from some preliminary studies that will allow you to further determine how these toxins function.

   Toxin 1:

   In cell culture experiments, you find that when you add the purified toxin to host cell monolayers, the host cells die. When you fluorescently label this toxin and add it to cultured cells, you find it as multimeric complexes within host cell membranes by fluorescence microscopy. Based on these results, answer the following questions:
   a. What type of toxin have you identified?
   b. How does your toxin function to kill host cells?

   Toxin 2:

   In cell culture experiments, when you add the purified toxin to host cell monolayers, the host cells die. The intoxicated host cells do not lyse, but the cells have collapsed and show a significant loss of water and ions. The toxin is translated and produced by the bacterium as a single polypeptide. However, cellular fractionation of the host cells into cytosolic and membrane fractions reveal that a portion of the toxin is present in the membrane fraction and another portion is in the cytosolic fraction. Based on these results, answer the following questions:
   c. What type of toxin have you identified?
   d. How does this toxin function to kill host cells?

2. Both diphtheria toxin and anthrax toxin have been used as prototypes for development of novel therapeutics.
   a. Diphtheria toxin was the first toxin to be exploited as an immunotoxin. Many other toxins have since been used as immunotoxins for specific purposes. Name at least two problems with the use of diphtheria toxin as an immunotoxin. What strategies have been employed to overcome these limitations?
   b. Describe how you might design an immunotoxin based on diphtheria toxin that might work to cure a tumor that overexpresses the ATR protein, which has an extracellular von Willebrandt factor A domain that binds to anthrax protective antigen, on its surface.

3. You have just isolated a new bacterium from a recent hospital outbreak that based on 16S rRNA comparison is closely related to *Acinetobacter baumannii. A. baumannii* is a Gram-negative, opportunistic, nosocomial (hospital-acquired) pathogen that is able to colonize patients in intensive care units, causing pneumonia, urinary tract infections, septicemia, or meningitis, depending on route

of infection. Epidemic strains of *A. baumannii* are often resistant to multiple drugs. Using transposon mutagenesis in a swine lung model of infection, you have identified two operons that may encode potential virulence factors.

a. One operon consists of two genes in tandem, which you have named *antBA*. Sequence comparison with protein databases reveals that *antA* encodes a protein of 25 kDa and has significant sequence similarity with the catalytic N-terminal 200 amino acid residues of diphtheria toxin, while *antB* encodes a protein of 10 kDa and has significant sequence similarity with the B subunit of cholera toxin. You suspect that the Ant protein is an AB-type toxin and decide to test this using a swine lung tissue cell model, and indeed Ant protein is cytotoxic to the cells. Further studies reveal that Ant action on lung cells requires a low pH-dependent step, since agents that prevent acidification, such as basic buffers, block toxin effects. You make antibodies against the culture filtrate from the bacterial cells and use the antisera in Western blots of the cell lysates from the bacterial cells, the media, and the intoxicated lung cells after separation of the proteins by SDS-PAGE. The results are shown in Figure 1. Provide a detailed explanation (with rationale) and interpretation for the results shown in the Western blot in Figure 1. Was your conclusion that Ant is an AB-type toxin correct? Explain.

b. Draw a diagram depicting a possible model that accounts for all of the experimental observations (i.e., predict the structure of the toxin and show the mode of action of the toxin). Be sure to justify your model (i.e., it must be consistent with your answer to part a).

c. The second operon consists of four genes in tandem, which you have named *anrCDEF*. The genes *anrCDE* have no sequence homology to any genes in the known databases and also have no obvious sequence motifs that could be used to determine their function. But, analysis of the protein sequence encoded by *anrF* revealed an interesting pattern. Shown in Figure 2 is a hydrophobicity plot of the predicted AnrF protein. Based on this information, you predict that AnrF might be a pore-forming toxin. What was your rationale for this prediction? Design an experiment that would allow you to determine if your prediction is correct.

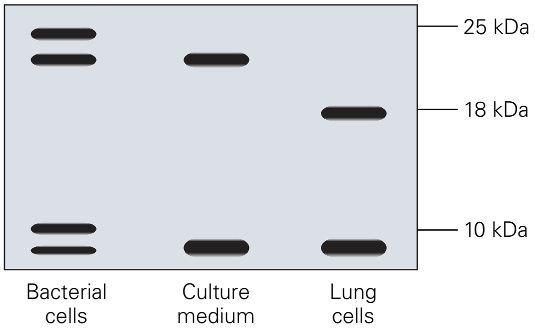

Figure 1. **Western blot using polyclonal antibodies against Ant protein.**

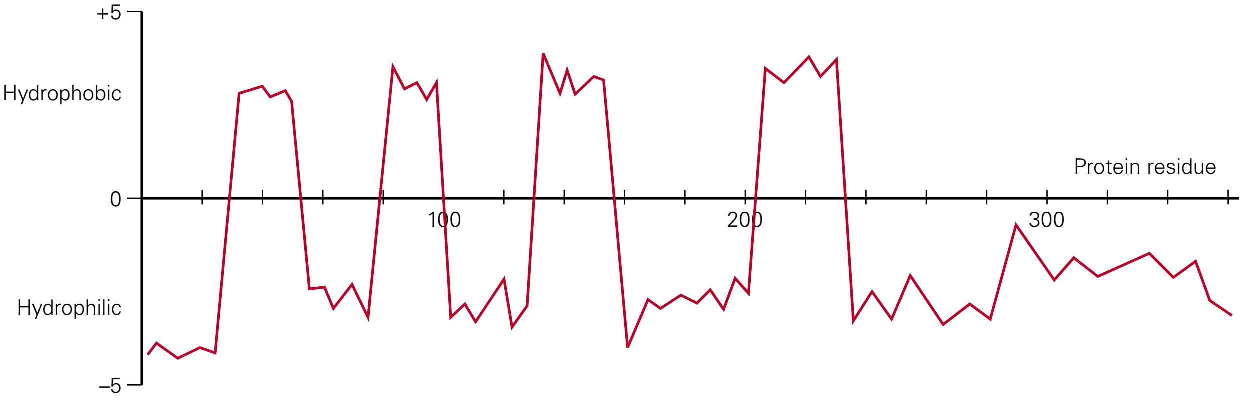

Figure 2. **Hydrophobicity plot for AnrF protein.**

13

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CHAPTER 13

Delivery of Virulence Factors

In the previous chapter, we discussed the various toxic factors used by pathogenic bacteria to modulate their environment and the host. However, simply producing these toxins is not enough. Unless they can be released into the environment or delivered into target cells, they are effectively useless. After all, what good is a bullet without a gun? Bacteria have evolved a number of mechanisms to mediate the secretion of various protein factors, which in turn allow them to attach to surfaces, to modulate the host immune system, or to kill neighboring cells. While all bacteria use protein secretion to control their surfaces and their environments to some extent, many bacterial pathogens are particularly aggressive with specialized injection systems to deliver toxic proteins directly into eukaryotic cells or even other competing bacteria.

Bacterial Secretion Systems and Virulence

To modulate their surroundings, bacteria have developed specialized secretion systems to transport proteins from the bacterial cytoplasm, where the proteins are produced, to the extracellular environment, where the proteins can take action. Although transport of proteins out of the cell is a normal process that all bacteria carry out, when these systems are used for the secretion of virulence factors, the secretion systems and their secreted proteins themselves become virulence determinants. Without a means to selectively deliver the adhesins, toxins, exoenzymes, proteases, and other virulence factors necessary for establishing infection, many bacteria are often not virulent to their hosts, even if they still produce potentially toxic factors. Indeed, most bacterial pathogens have evolved specialized, and often dedicated, secretion systems specifically for the transport of these virulence factors out of the cytoplasm and onto the bacterial cell surface, into the medium, or directly into target cells.

Because Gram-negative bacteria have two lipid bilayers (see Box 1-4) across which proteins must be transported in order to be secreted into the extracellular medium, the protein secretion systems of Gram-negative bacteria are more numerous, more complicated, and more diverse than those of Gram-positive bacteria. Exporting proteins from the cytoplasm across the cell membrane of Gram-positive bacteria or the inner membrane of Gram-negative bacteria is mediated by some secretion systems that are common to both, so we will begin with those. In the case of Gram-negative bacteria, the secreted proteins end up in the periplasm, and additional secretion systems must be utilized to further transport the proteins across the outer membrane. Gram-negative bacteria also have other specialized secretion systems that do not utilize the general secretion systems common to both types of bacteria. These additional secretion systems have different sets of secretion proteins that enable transport across both the inner and outer membranes. Finally, we will consider features of the protein export systems used by several Gram-positive bacteria, including secretion systems that allow protein effectors to be transported through hydrophobic surface layers.

Common Secretory Systems

The General Secretory (Sec) System

The **general secretory (Sec) system** is common to Gram-positive and Gram-negative bacteria and has been well characterized in *Bacillus subtilis*, *Escherichia coli*, and a number of other bacterial species. The secreted proteins are synthesized as precursors with a 15- to 26-amino acid **signal sequence** (also called a **leader sequence**) at the N terminus, which contains 10 to 12 hydrophobic residues preceded by one or two charged residues and followed by a protease cleavage site. The signal sequence is cleaved by a leader peptidase at this site during the process of transport from the cytoplasm across the membrane. The hydrophobic residues of the leader sequence trap the cleaved peptide in the membrane, while the rest of the protein is released. Mutations in the protease cleavage site do not prevent transport across the membrane but instead result in the protein being tethered to the membrane instead of being released into the medium or periplasm. In some cases, lipids are attached to proteins at a cysteine residue located downstream of the cleavage site. Following export and cleavage by a different leader peptidase than the one used for other exported proteins, these lipoproteins remain anchored to the cell inner membranes and, in Gram-negative bacteria, some lipoproteins are moved to the outer membrane. In Gram-positive bacteria especially, lipoproteins are exposed on the surface and can act as surface antigens to the immune system.

The signal sequence is required for secreted proteins to be recognized and transported by the Sec system (Figure 13-1A). The hallmark of the Sec system is that it exports unfolded proteins. The vast majority of this secretion occurs posttranslationally, for which the secretion substrate is mostly or fully synthesized first, but for some substrates (particularly membrane-localized proteins) this process occurs cotranslationally. In the posttranslational pathway, two cytoplasmic proteins are responsible for binding and directing the protein to be secreted. **SecB** is a tetrameric protein chaperone that binds to protein substrates containing signal peptides as they exit the ribosome during translation. In Gram-negative bacteria, the SecB tetramer contains a chamber that is thought to bind segments of the proteins and keep them unfolded until they can be delivered to **SecA**. In Gram-positive bacteria, SecB homologs are missing and it is likely that other proteins act as chaperones to keep proteins unfolded before delivery to SecA. The SecA dimer is a molecular motor whose ATPase activity provides part of the energy for the translocation process, while the rest of the energy seems to be provided by the proton motive force across the membrane.

SecA binds to the heterotrimeric **translocase complex** composed of **SecYEG**, which forms a **protein-conducting channel** through the cellular membrane. SecB delivers the unfolded protein substrate to the ADP-bound SecA-SecYEG complex, while additional proteins (SecD, SecF, and YajC) help stabilize the entire complex. The N terminus of the protein containing the signal peptide is then threaded through a pore made of SecA-SecYEG and remains bound to SecA at its interface with SecYEG. The bound ADP is exchanged for ATP, which induces a conformational change that releases SecB from the complex. ATP hydrolysis is then coupled to the driving of the polypeptide chain through the channel in SecYEG. The signal peptide remains tethered in the complex and is cleaved by the activity of a leader peptidase, after which the peptide is released and refolds. ADP is then exchanged again for ATP, and the binding and hydrolysis cycle is repeated until the entire protein is secreted across the membrane. In Gram-positive bacteria, the mature protein folds as it emerges through SecYEG and is released to the surfaces of the bacteria or directly into the medium. In Gram-negative bacteria, the transported protein folds in the periplasmic space, where it may remain or be transported across the outer membrane by one of the specialized transport mechanisms described later in this chapter.

The Accessory Secretory (Sec) System

In some Gram-positive bacteria, including species of *Streptococcus*, *Listeria*, and *Mycobacterium*, there is a second independent export system, called the **accessory Sec system** (Figure 13-1B), that exports specific virulence factor proteins to the cell surface. In these bacterial species, there are two SecA proteins: SecA1, which is closely related to the canonical (i.e., usual) SecA of other systems and functions in the normal protein export pathway, and SecA2, which functions in the accessory export pathway, often with a homolog of the SecY translocase (SecY2) and other accessory Sec proteins (Asp1 through Asp5). These SecA2-SecY2 accessory systems export serine-rich glycosylated virulence factor proteins that cannot be exported through the canonical SecA export system.

The Cotranslational Signal-Recognition Particle (SRP) System

Many proteins in Gram-positive and Gram-negative bacteria remain embedded within the cell membrane after being translocated across it, where they play various roles in ion and nutrient transport, metabolism, and signaling. These integral membrane (**polytopic**) proteins consist of several hydrophobic transmembrane-spanning α-helices joined together by hydrophilic linker regions that face into the cytoplasm or outside of the cell membrane. While these proteins also utilize the SecYEG complex, they are secreted cotrans-

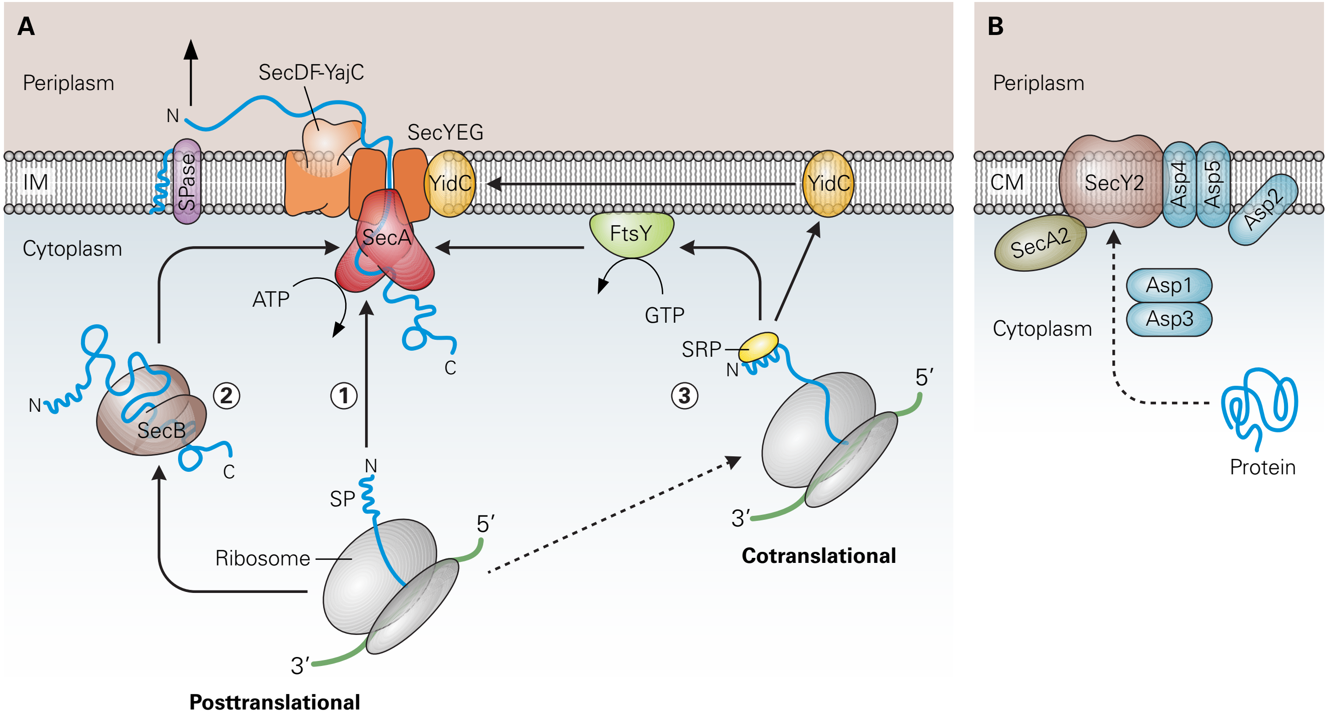

Figure 13-1. The general secretory (Sec) system. The Sec system is common to both Gram-negative and Gram-positive bacteria. **(A)** During posttranslational secretion, SecA binds the signal peptide (SP) of the secretion substrate protein and the SecYEG translocase in the inner membrane (IM). SecA undergoes an ATP-dependent conformational change that drives translocation through the pore of the translocase. Chaperones such as SecB can help the translated proteins remain unfolded before being delivered to the SecA-SecYEG complex. SecDF-YajC helps stabilize this process. During cotranslational secretion, SRP binds the SP of the nascent peptide as it emerges from the ribosome. The SRP-bound peptide is then delivered to the SecYEG complex with the help of the SRP receptor FtsY. Some proteins go through an alternate route involving YidC alone or in concert with the SecYEG complex. A signal peptidase (SPase) then cleaves the signal sequence, allowing the mature protein to be released. Data from Collinson I, Corey RA, Allen WJ. 2015. *Philos Trans R Soc Lond B Biol Sci* 370. **(B)** The specialized accessory Sec system found in some pathogens.

lationally (unlike the standard posttranslational pathway) because transport is coupled with production by the ribosome (Figure 13-1A). As polytopic proteins exit the ribosome, they are positioned such that their signal peptides bind to the **signal-recognition particle (SRP) complex**, which consists of 4.5S RNA and a protein called Ffh. The SRP complex then docks to the membrane-bound SRP receptor protein, called **FtsY**, which hydrolyzes GTP and delivers the signal peptide to the SecYEG complex. Further translation by the ribosome drives the nascent membrane protein from the SecYEG complex into the cellular membrane. This cotranslational protein export system involving the SRP is similar to a mechanism used by eukaryotic cells. For a subset of polytopic membrane proteins, translocation involves a separate protein called **YidC**, which acts by itself or together with the SecYEG complex to insert the protein into the membrane (Figure 13-1A).

Finally, there are many bitopic membrane proteins that contain a single transmembrane α-helix separating the cytoplasmic and extracellular domains of the protein. Bitopic membrane proteins play many important cellular functions, especially in the biosynthesis of bacterial peptidoglycan cell walls and in transmitting extracellular signals to the cytoplasm of the cell. When the transmembrane helix of a bitopic protein is near the N terminus of the protein (i.e., when the cytoplasmic domain is small), membrane insertion and export of the large C-terminal domain across the membrane is complex and involves both the SRP-YidC system and the SecA system, and the SecA system is driven by the proton motive force across the cell membrane.

The Twin-Arginine Transport (TAT) System

The **twin-arginine transport (TAT) system** is a protein export pathway found in both Gram-positive and Gram-negative bacteria. The unique feature of the TAT system is that it is dedicated to the transmembrane translocation of proteins that are fully folded in the cytoplasm (Figure 13-2). Some of the proteins that are exported by the TAT system contain cofactors, and cytoplasmic folding may optimize insertion of the cofactors in these proteins. However, not all TAT substrates contain cofactors, nor is the TAT system found in all bacterial species. Proteins are targeted to the membrane-embedded TAT translocase by N-terminal twin-arginine signal sequences. The TAT signal sequence includes a polar

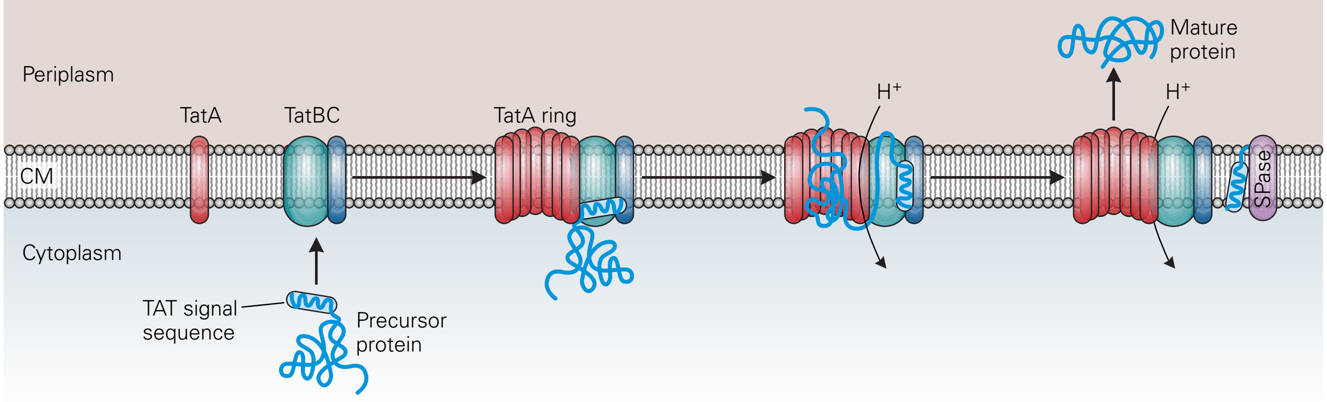

Figure 13-2. The TAT system. Shown is a model for the membrane transport of proteins through the TAT system. Precursor proteins bind to TatBC through their TAT signal sequences. TatA is recruited to the resulting complex and forms a large pore, then a major conformational change, possibly induced by a proton electrochemical gradient, induces transport of the protein across the membrane. The TAT signal sequence is then cleaved by a surface signal peptidase and the mature protein is released. Data from Goosense VJ, van Dijl JM. 2017. *Curr Top Microbiol Immunol* **404:**69–94, with permission.

N-terminal region of variable length, followed by a region bearing the motif SRRXFLK, and then a hydrophobic region of 12 to 20 amino acid residues, sometimes with a few basic residues. The TAT translocase consists of three proteins, **TatABC**. The TAT signal sequence is recognized by TatBC, which induces assembly of the TatA proteins to form a large pore complex. The proton motive force across the membrane is thought to energize the transport step. Following transport, the TatABC complex dissociates again. Fusion of a TAT signal sequence to the amino terminus of recombinant proteins can be used as a method to get folded proteins exported out of the cytoplasm of Gram-positive or Gram-negative cells that have TAT systems.

Secretion Systems Specific to Gram-Negative Bacteria

Because of their outer membrane, Gram-negative bacteria have at least six different mechanisms for secretion (see Table 13-1). These secretion systems, termed type 1 through type 6 (also denoted type I through type VI), come in two flavors: those that depend on the general Sec system for transport from the cytoplasm into the periplasm (Sec-dependent systems type 2 and type 5) and those that do not (Sec-independent systems type 1, type 3, type 4, and type 6).

Sec-Dependent Secretion Systems

T2SS. Proteins secreted through the type 2 secretion system (T2SS) use the general Sec system to reach the periplasm. Once folded, they then traverse the outer membrane through a channel made by special pore-forming proteins (Figure 13-3). The T2SS involves a total of 12 to 14 proteins that are encoded by a cluster of genes. The T2SS apparatus is evolutionarily related to other bacterial systems, including the type IV pili biogenesis system (not to be confused with the type 4 secretion system discussed later), archaeal flagella, filamentous phages, and the competence system for DNA uptake in Gram-positive bacteria. The first T2SS characterized was the *pul* system from *Klebsiella oxytoca*. Other examples are the *out* system of *Dickeya dadantii*, the *xcp* system of *Pseudomonas aeruginosa*, the *exe* system of *Aeromonas hydrophila*, the *xsp* system of *Xanthomonas campestris*, and the *eps* system of *Vibrio cholerae*. The T2SS is used to secrete the A and B portions of some AB toxins (see chapter 12). For example, the ADP-ribosylating toxin from *P. aeruginosa* (ExoA) is exported through the *xcp* system, and cholera toxin is exported through the *eps* system of *V. cholerae*.

The T2SS consists of two large complexes: an outer membrane complex forming a gated channel through which the substrates are translocated and an inner membrane complex that forms a platform from which a filamentous pseudopilus extends. The pseudopilus, unlike fully formed pili, does not extend outside the cell and remains fully contained within the periplasmic space. T2SS substrates are first delivered into the periplasm by either the Sec or TAT secretion systems and position themselves between the outer membrane pore and the pseudopilus extending from the inner membrane platform. As the pseudopilus extends, it forces the substrate through the outer membrane pore in a piston-like manner.

T5SS. In the type 5 secretion system (T5SS), the protein to be exported, called an **autotransporter**, is delivered to the periplasm via the general Sec system, where it then transports itself across the outer membrane. The autotransporter is made as a single precursor protein with multiple domains: an N-terminal Sec signal sequence, a passenger (P) domain, a linker (L) region, and a C-terminal β domain. Once the

Table 13-1. Summary of bacterial secretion systems

SECRETION SYSTEM	CHARACTERISTIC FEATURES	SECRETION SIGNAL	SUBSTRATES
General Secretion Systems			
Sec	The General Secretory Pathway (GSP), or Sec, is found in the cytoplasmic membrane of all bacteria. Chaperone proteins guide substrate proteins to a membrane-localized translocon complex, which transports them in an unfolded state from cytoplasm to the extracellular milieu in Gram-positive bacteria or to the periplasm in Gram-negative bacteria.	N-terminal signal peptide	Various surface and secreted proteins
TAT	The TatABC translocon is found in the cytoplasmic membrane of both Gram-positive and Gram-negative bacteria. It transports fully folded proteins, including bound cofactor ligands, from the cytoplasm to extracellular milieu in Gram-positive bacteria or to the periplasm in Gram-negative bacteria.	N-terminal signal peptide often containing two arginine residues	Various surface and secreted proteins; often fully folded and sometimes as multiprotein complexes
Gram-Negative Secretion Systems			
Sec-dependent			
T2SS	Two-step secretion machinery. The first step utilizes Sec (or sometimes TAT) systems to transport proteins into the periplasm. Proteins or multimeric complexes assembled in the periplasm are transported to extracellular milieu by the outer-membrane components of the T2SS that are evolutionarily related to pilus biogenesis machinery.	Sec or TAT signal for periplasmic delivery; T2SS signal remains unknown	In pathogenic Gram-negative bacteria, exported proteins are often hydrolyzing enzymes or toxins (e.g., cholera toxin).
T5SS	The substrate and the outer membrane secretion pore form the single chain auto-transporter protein. Two-step secretion of the autotransporter utilizes the Sec system to reach the periplasm. There, its translocation domain inserts itself into the outer membrane to form a pore through which the rest of the protein can be translocated.	Sec signal for periplasmic delivery; N-proximal secretion domain drives its own translocation across the outer membrane.	Examples include IgA and IgG proteases, *H. pylori* vacuolating cytotoxin (VacA), and *Bordetella* pertactin (surface-localized adhesin).
Sec-independent			
T1SS	Found in all bacteria. It is an ABC transporter comprised of three proteins: ATP-binding cassette protein in inner membrane; outer membrane channel-forming protein; and inner-outer membrane fusion protein spanning the periplasm. Unfolded proteins are exported from bacterial cytoplasm directly into extracellular milieu.	C-terminal signal peptide	In pathogenic Gram-negative bacteria, exported proteins are often associated with nutrient acquisition (iron transport) or virulence (pore-forming hemolysins and multidrug efflux pumps). It is capable of transporting very large proteins (up to 800 kDa).
T3SS	Elaborate multiprotein complexes, spanning the inner membrane, periplasm, and outer membranes, found in many Gram-negative bacteria. The injectisome serves as a specialized protein delivery apparatus for transferring unfolded effector proteins from the bacterial cytoplasm into the plasma membrane or cytosol of target eukaryotic cells. Effector proteins are folded after the delivery process in the target cell. It is evolutionarily related to flagella assembly and basal body motor apparatus.	The N-terminal signal motif on the effector substrates are recognized by two classes of cytoplasmic chaperones.	Effector proteins from pathogenic bacteria (e.g., *E. coli, Yersinia, Salmonella, Shigella, Bordetella, Pseudomonas, Xanthomonas, Ralstonia, Erwinia*, etc.) modulate or subvert specific host functions through enzymatic activity on host molecules or through binding interactions with host targets.

(*continued*)

Table 13-1. **Summary of bacterial secretion systems (*continued*)**

SECRETION SYSTEM	CHARACTERISTIC FEATURES	SECRETION SIGNAL	SUBSTRATES
T4SS	Specialized secretion machinery found in Gram-positive and Gram-negative bacteria and some archaea that spans the inner and outer membranes and inserts into target bacterial or eukaryotic cell membranes. Delivers folded or unfolded effector proteins or DNA directly from the bacterial cytoplasm into eukaryotic host cells or other bacterial cells. Evolutionarily related to DNA conjugation systems. Some T4SSs utilize a Sec system to first transport proteins into periplasm.	Chaperone dependent	Effector proteins from intracellular pathogens (*Legionella* and *Brucella*) used for survival inside host cell vacuoles; *Bordetella pertussis* toxin involved in virulence; transfer of pathogenic conjugative Ti plasmid DNA and effector proteins from *Agrobacterium tumefaciens*
T6SS	Specialized secretion machinery found widely in Proteobacteria. Evolutionarily and mechanistically related to contractile bacteriophage tails. Secreted substrates decorate the tip or interior of an inner tube. Contraction of the sheath proteins surrounding this inner tube results in the inner tube being ejected from the cell into target cells.	Substrates are attached to the tip of the inner tube through covalent and noncovalent interactions with the tip complex or incorporation into the inner tube via Hcp.	Folded effector proteins conferring toxicity against both eukaryotic and bacterial targets
Gram-Positive Secretion Systems			
Sec-dependent			
Different arrangements of SecA-SecYEG transporter	Depending on species, SecA-SecYEG often localize to division septa and equatorial rings; sometimes they localize uniformly over cell peripheries or in spirals along the surfaces of rod-shaped cells; sometimes they are found localized as a single microdomain (called an ExPortal); often the SrtA sortase and quality-control proteases are coordinated with SecA-SecYEG localization.	Sec secretion system	All exported proteins, except T7SS substrates, including toxins and virulence factors
Accessory Sec system	The SecA2/SecY2 system exports modified virulence factors that are not substrates for the normal SecA-SecYEG transporter.	Unusually long, tripartite signal peptides that prevent SecA export	Glycosylated serine-rich virulence factor proteins
Sortase system	Sortases covalently attach secreted proteins to the cell wall following secretion across the cytoplasmic membrane via the canonical Sec system. Sortase (SrtA) cuts between TG of the LPXTG motif and forms a covalent link with the C-terminal T and cysteine residue of SrtA, which then transfers the protein to an amino group of the cell wall lipid II, which is embedded in the cell wall.	N-terminal Sec secretion signals plus 30–40 residue C-terminal cell wall sorting signal that contains a pentapeptide cleavage site motif LPXTG and a hydrophobic domain	All exported proteins that are destined for the outer cell wall surface, such as pili or other adhesins, enzymes (proteases), and proteins A and G in staphylococci and streptococci, respectively
Cytolysin-Mediated Translocation (CMT)	CMT is an Sec/SRP-dependent specialized host uptake mechanism in *S. pyogenes* (Group A strep); secreted streptolysin O (SLO) in the presence of the NAD-glycohydrolase toxin (SPN) binds to host cell membranes independent of cholesterol, resulting in the delivery of SPN to the host cell cytoplasm, possibly by vesicle formation; SLO-SPN complex in host membranes inhibits pore formation by SLO.	Sec/SRP secretion signals	Delivery of the SPN toxin to the cytoplasm of host cells adjacent or adhered to *S. pyogenes* cells
Sec-independent			
T7SS	Specialized secretion machinery discovered and most studied in high-GC Gram-positive bacteria that produce a complex waxy surface layer, such as mycobacterial species; may also be present in some low-GC Gram-positive pathogens. Folded proteins from the bacterial cytoplasm are targeted to an ATPase pump protein that contacts a series of other proteins in the inner membrane. These complexes transport the substrate proteins through the inner membrane and mycomembrane surface barriers.	The C-terminal domain in the substrates acts as a secretion motif that is cleaved off at the cell surface.	In *Mycobacteria*, EsxA and EsxB pore-forming toxins are used to escape phagosomes; they may also be involved in transfer of chromosomal DNA fragments.

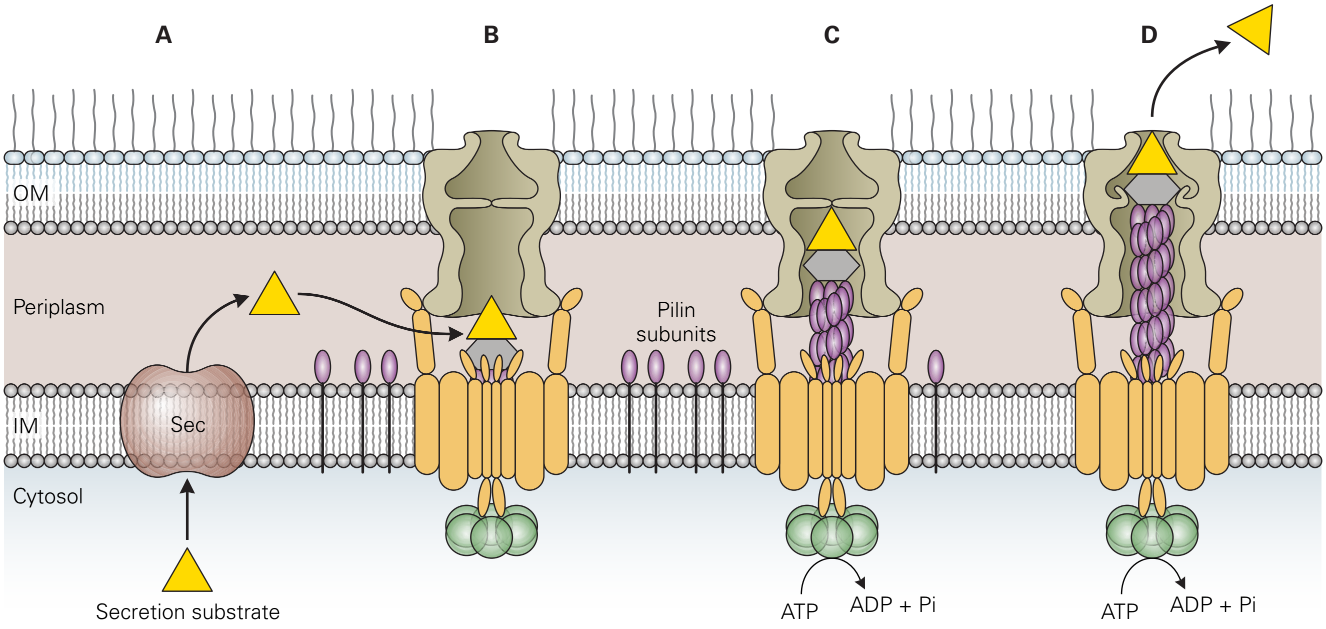

Figure 13-3. The T2SS. Shown is a model for the mechanism of protein secretion by the Sec-dependent T2SS. (**A**) Secretion substrates are delivered into the periplasmic space by Sec or TAT systems. (**B**) The inner membrane complex assembles opposite the outer membrane pore. The pseudopilin extends from the inner membrane complex, while the secretion substrates bind to the tip of the pseudopilin. (**C**) ATP hydrolysis by a cytoplasmic ATPase drives incorporation of pilin subunits embedded in the inner membrane into the pseudopilus. (**D**) Extension of the pseudopilus pushes secretion substrates out of the cell. Adapted from Korotkov KV, Sandkvist M, Hol WG. 2012. *Nat Rev Microbiol* **10:**336–351, with permission.

protein enters the periplasm via the Sec secretion system, the signal sequence is cleaved. The β domain then inserts into the outer membrane and forms a pore structure through which the remaining domains translocate to the bacterial cell surface. At this point, the protein may or may not undergo further processing. For some autotransporters, autoproteolysis (catalyzed by a protease domain contained within the passenger domain) releases the other secreted domains into the medium. For others, autoproteolysis does not occur and the protein remains associated with the bacterial cell surface.

The mechanism of secretion by autotransporters (Figure 13-4) was discovered first for the *Neisseria* immunoglobulin A (IgA) protease, which was the only gene product, other than the proteins making up the general Sec apparatus, necessary for secretion of active IgA protease from *E. coli* transformed with a plasmid carrying the IgA protease gene. Other examples of autotransporters are the IgA protease from *Haemophilus influenzae*, the serine protease from *Serratia marcescens*, the vacuolating cytotoxin (VacA) from *Helicobacter pylori*, and pertactin, the adhesin from *Bordetella* used in current DTaP vaccines (see chapter 18).

Sec-Independent Secretion Systems

T1SS. Proteins secreted by the type 1 secretion system (T1SS) cross directly from the cytoplasm to the cell surface, bypassing the general Sec system and the periplasm. The T1SS is capable of transporting proteins up to 800 kDa in size out of the cell in only a few seconds. The T1SS consists of a three-protein complex that spans the inner membrane, the periplasm, and the outer membrane and delivers protein substrates directly into the medium in a single step. The component that spans the inner membrane is an **ATP-binding cassette (ABC) transporter** whose ATPase activity supplies the energy for traversing the membrane. The secreted protein binds to the ABC transporter protein via a C-terminal signal sequence of approximately 50 amino acid residues, which contains an amphipathic helix with a glycine-rich GGXGXD sequence motif that is repeated up to 36 times. Unlike the Sec signal sequence, the signal sequence for the T1SS is not cleaved during export. The component that spans the outer membrane is also a pore-forming protein made up of three monomers. A periplasmic-connecting protein called an **accessory factor** (sometimes called a membrane fusion protein) holds the two pore-forming components together so that the secreted protein can be funneled directly from the cytoplasm into the medium. The α-hemolysin (HlyA) from *E. coli* is secreted by a T1SS made up of the ABC transporter HlyB, the accessory factor HlyD, and the outer-membrane pore protein TolC (Figure 13-5). TolC is also utilized by the **multidrug efflux system** in *E. coli*, which consists of TolC plus an ABC transporter, AcrB, and an accessory factor, AcrA.

T3SS. One of the most exciting developments in the toxin delivery area was the discovery that some bacteria do not

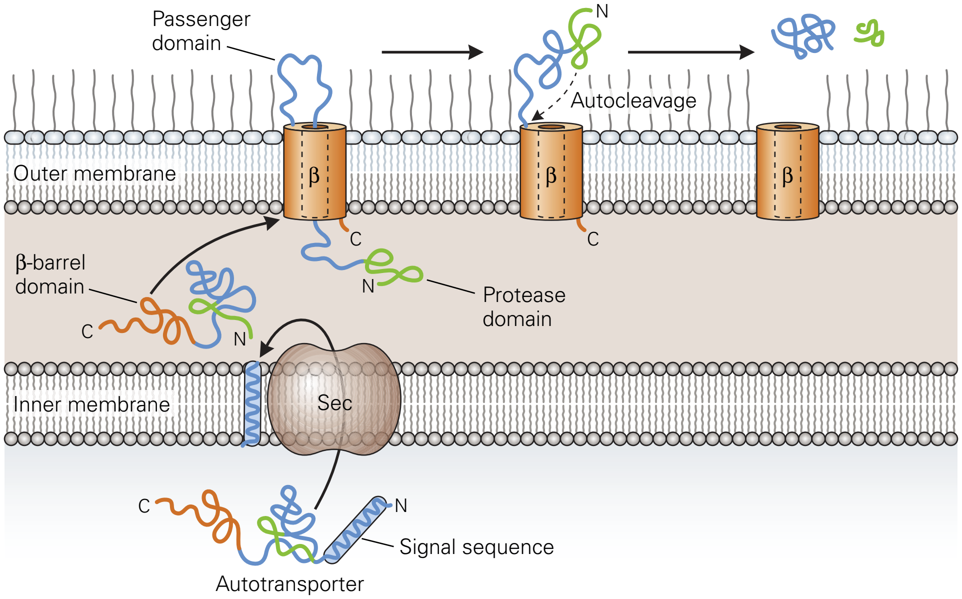
Passenger domain
N
Autocleavage
β
β
β
Outer membrane
C
C
β-barrel domain
N
Protease domain
C
N
Sec
Inner membrane
C
N
Signal sequence
Autotransporter

Figure 13-4. **The T5SS.** Shown is a model for the mechanism of protein secretion by the Sec-dependent T5SS. The autotransporter protein is delivered into the periplasmic space via the general Sec system. Once in the periplasm, the β domain of the autotransporter inserts into the outer membrane and forms a pore through which the other domains of the protein traverse. In some autotransporters, autoproteolytic cleavage releases the other domains from the β domain. In other autotransporters, cleavage does not occur and the protease and other domains remain attached to the β domain. Data from Fan E, Chauhan N, Udatha DB, Leo JC, Linke D. 2016. *Microbiol Spectr* **4(1):**VMBF-0009-2015.

simply excrete their toxins into the extracellular medium but rather inject their toxic effector proteins directly into the eukaryotic cell cytosol. This contact-dependent toxic effector protein delivery system, called the type 3 secretion system (T3SS), was first described in *Yersinia pestis*, the causative agent of bubonic plague, but its mechanism of toxic effector protein delivery has proved to be surprisingly ubiquitous. In addition to *Yersinia*, a large number of pathogens (e.g., path-

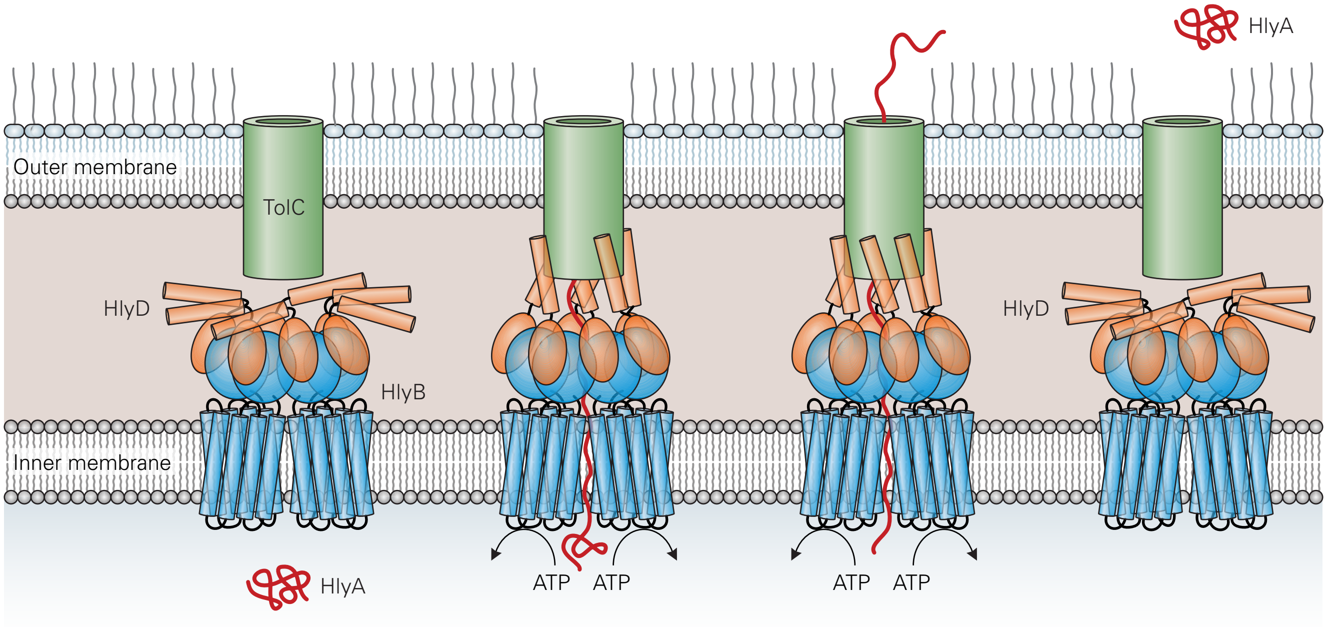
HlyA
Outer membrane
TolC
HlyD
HlyD
HlyB
Inner membrane
HlyA
ATP
ATP
ATP
ATP

Figure 13-5. **The T1SS.** Shown is a model for the mechanism of protein secretion by the Sec-independent T1SS from *E. coli* that secretes α-hemolysin (HlyA). This T1SS consists of three proteins: an inner membrane (IM)-spanning ABC transporter (HlyB); a periplasmic-connecting protein (accessory factor; HlyD); and an outer membrane (OM)-spanning pore protein (TolC). Data from Phillips JL, Gnanakaran S. 2015. *Proteins* **83:**46–65.

ogenic *E. coli*, *Salmonella*, *Shigella*, *Bordetella*, *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Chlamydia*) have been found to contain T3SS apparatuses. The genes encoding the T3SS are often located in clusters on large **pathogenicity islands (PAIs)**, suggesting that these systems may have moved horizontally between different bacterial species.

The T3SS apparatus is an elaborate complex of over 20 proteins that forms a needlelike structure (often referred to as an **injectisome**) that acts like a molecular syringe to deliver proteins directly into the host cell upon intimate (tight) contact with the host cell surface. As for the flagellar system, the proton motive force (pmf) provides the energy for the rate-limiting step of the T3SS transport of proteins through the needle of the injectisome. The overall morphology and organization of the virulence-associated T3SS appear to have evolved from that of the bacterial flagellar assembly apparatus (Figure 13-6). Both flagella and the T3SS have a large basal body complex embedded in the inner membrane. Within this complex are ATPases that facilitate release of the chaperones and unfolding of the effectors for secretion. Refolding upon exit of the needle is thought to also help in facilitating the transport process, as well as several switch proteins that regulate which substrates are secreted. Attached to the basal body is a channel that spans the periplasmic space and outer membrane. This channel is significantly different between flagella and the T3SS. Although the precise reason for this difference is unclear, it is likely related to the need for this channel to support a large rotating flagellar body, whereas the T3SS injectisome needs to be able to seal itself off before the needle structure is assembled to secrete the effector proteins upon contact with the host cell (Figure 13-7A).

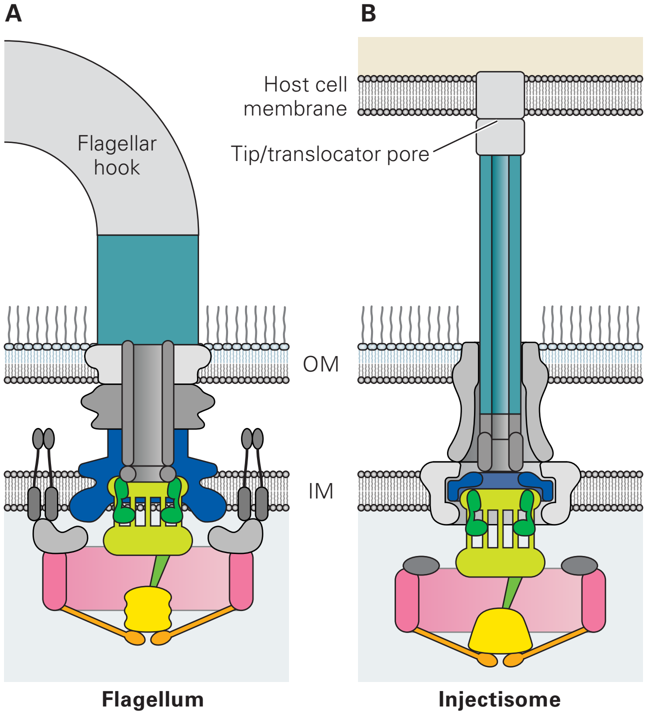

Figure 13-6. Comparison of the flagellar assembly with the T3SS. The overall structure and organization of the bacterial flagellar apparatus and the T3SS injectisome are highly similar, with many protein components sharing homology. Both structures consist of inner and outer membrane ring structures, proton motive force (pmf) drives translocation, a periplasm and outer membrane-spanning channel, and a helical extracellular filament structure. The tip of the T3SS filament forms a translocator pore in the host cell membrane that translocates T3SS effector proteins directly into the cytoplasm of the host cell. In the illustration, corresponding homologs are indicated with matching colors. Gray-colored components are specific for their respective systems. Adapted from Diepold A, Armitage JP. 2015. *Philos Trans R Soc Lond B Biol Sci* **370(1679)**, with permission.

Once the basal membrane complex is assembled, both flagella and T3SS begin secreting the structural components that comprise the flagellar body and needle, respectively. The length of the flagella and T3SS needle are both controlled by a so-called tape measure protein (Figure 13-7B). For the T3SS, when the needle reaches the necessary length, components of the tip complex are secreted and assembled at the end of the needle structure. Finally, after contact with a target host membrane occurs, secreted toxins are threaded through the needle structure into the host cell cytosol through a translocation pore that forms in the host cell membrane. These exported toxins have specific bacterial cytoplasmic chaperones that bind them and guide them to the injectisome for secretion and translocation.

When one considers host defenses, there are clear advantages to directly delivering toxic effector proteins into the host cytosol compared to simple toxin excretion and intoxication as an AB type toxin. One of the key defense mechanisms for combating bacterial toxins (chapter 12) is the production of neutralizing antibodies that prevent toxins from binding to their target cells or that obstruct the toxin's enzymatic active site. By being directly injected into the host cell, effector proteins avoid ever being truly extracellular and therefore never come into contact with these neutralizing antibodies.

The discovery that toxic effector proteins could be directly injected into the cytosol of host cells explained a long-existing conundrum about the function of a set of putative virulence factors called exoenzymes. These enzymes were often found in the extracellular medium and were thought to be the catalytic A subunit of AB toxins for which the cognate B subunit was missing. Initially, it was assumed that the missing B subunits of these so-called orphan A subunits existed but simply had not yet been identified. Note that at the time, the genome sequences of pathogens were few and far between, so identifying toxin components by their genes was still almost a decade away. With the discovery of the T3SS, it became clear that these toxin B subunits were not missing because they had not been

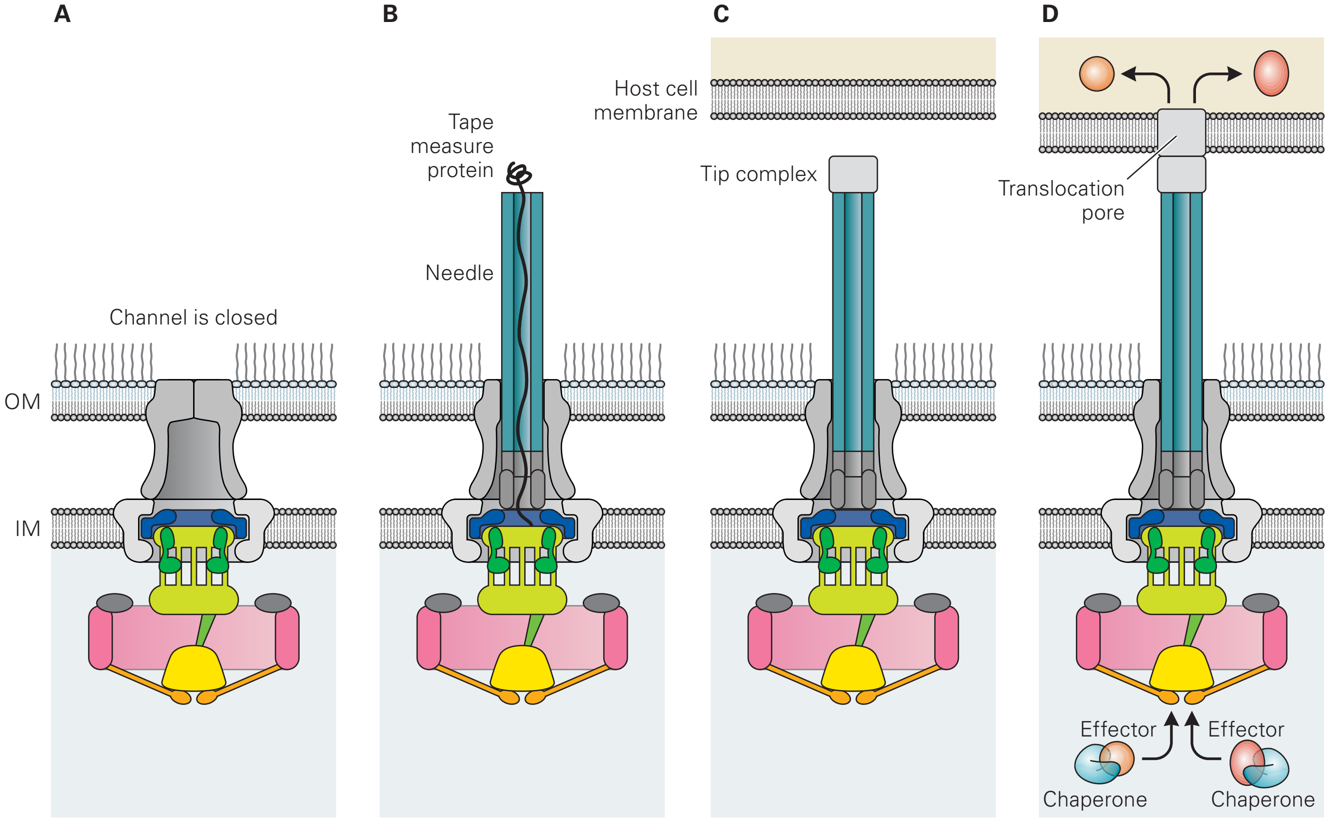

Figure 13-7. Assembly of the T3SS. Shown is a model of how the T3SS apparatus is assembled. (A) The basal structure is first assembled in the inner and outer membranes with the outer membrane channel structure remaining closed. **(B)** Needle components are secreted and assembled. The tape measure protein controls the needle length. **(C)** After the needle is fully assembled, tip complex components are secreted and assembled. **(D)** Upon contact with the host cell membrane, the translocator pore is formed in the host cell membrane. Chaperone proteins guide the T3SS effector proteins to the T3SS apparatus, and the effectors are released from the chaperone and unfolded through an ATP-driven process and secreted through the needle by the proton motive force. Adapted from Diepold A, Armitage JP. 2015. *Philos Trans R Soc Lond B Biol Sci* **370(1679)**, with permission.

found, but rather they were not actually needed for translocation into target cells in the first place. These exoenzymes, while lacking B subunits, still have profound effects on the host cell when they are expressed through transfection of mammalian expression plasmids or directly microinjected via needles. As such, they are now most often referred to as **effector proteins** instead of exoenzymes.

When the T3SS was discovered, two key questions emerged. First, are effector proteins really injected directly from the bacterial cytoplasm into the cytosol of host cells? And second, which of the many exoenzymes secreted from the bacterial cell are also effector proteins that are delivered directly into the host cell? It was known that simply adding effector proteins, even at relatively high concentrations, to the medium was not enough to cause cytotoxicity in host cells; however, at the time (circa 1994), the prevailing model was that the effector proteins were the A subunits of AB toxins and that the “missing” B subunits had simply not been identified yet. Convincingly demonstrating that a specific effector could be delivered directly into a target cell would be a big step toward addressing these questions and ruling out the need for a B subunit.

Two key experiments, performed by researchers working on *Yersinia* pathogenesis, gave the first clear evidence for direct translocation of the effector protein YopE from the bacterial cell into the eukaryotic cell. YopE, along with YopH, YopT, and YpkA (also known as YopO), acts to prevent phagocytosis by host macrophages and polymorphonuclear leukocytes by targeting host proteins involved in actin cytoskeletal rearrangement. YopE disrupts the cell actin microfilament structure and causes rounding of host cells by acting as a GTPase-activating protein (GAP) to inhibit the RhoA family of small GTPases that regulate actin cytoskeletal assembly.

In the first study, confocal fluorescence microscopy was used to show that the bacteria remain attached at the eukaryotic cell surface, while translocated YopE is found only within the host cell cytosol. In the second study, the researchers constructed a recombinant *Yersinia* strain that produced YopE fused to a reporter enzyme, the **calcium-calmodulin-dependent adenylate cyclase (Cya)** toxin

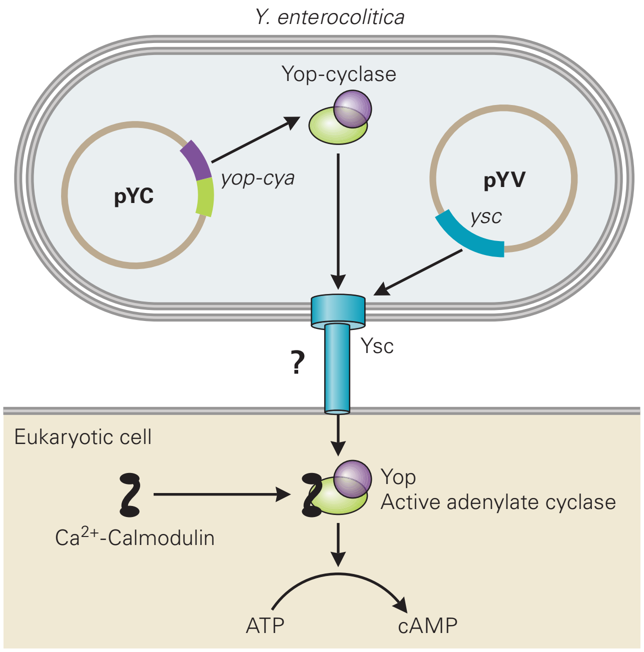

Figure 13-8. Effector-reporter strategy for identifying translocated Yop proteins. The Yop-Cya reporter system consists of the adenylate cyclase (Cya) toxin from *Bordetella*, which converts ATP into cAMP only in the presence of the calcium-binding protein calmodulin. Since calmodulin is found only in the cytosol of the host cell, cAMP production is increased only when the Yop-Cya fusion protein is delivered into the cytosol. Incubation of a *Yersinia enterocolitica* strain producing the YopE-Cya protein with host cells resulted in accumulation of cAMP, but only when the Ysc T3SS was also expressed in the bacteria and when the cells were in direct contact with each other via an adhesin. Adapted from Sory MP, Cornelis GR. 1994. *Mol Microbiol* **14:**583–594, with permission.

from *Bordetella pertussis*, which converts ATP into cyclic AMP (cAMP) (Figure 13-8). Because bacteria do not have calmodulin (which is located only in the cytosol of eukaryotic cells), cAMP production could occur only if the Cya protein is delivered into the host cell. Mutants lacking components of the T3SS did not allow Cya delivery to the host cell and thus showed no cAMP production. The researchers further demonstrated that bacterial adhesion to the host cells was necessary because mutants lacking the adhesin did not cause cAMP production. Moreover, cAMP production was not due to uptake of the bacteria or YopE-Cya fusion proteins into the cells because inhibitors of actin polymerization (such as cytochalasin D, which blocks phagocytosis) had no effect on cAMP production.

Additional approaches were later developed to further confirm effector delivery into host cells. One such approach was to use subcellular fractionation and immunoblotting techniques to determine the location of translocated effectors. This subcellular fractionation is achieved by treating cells with **digitonin**, a nonionic detergent that selectively permeabilizes the eukaryotic plasma membrane but not the nuclear membrane. After digitonin treatment, nuclear and cytosolic fractions can be separated by centrifugation. Immunoblotting with anti-YopM or anti-YopE antibodies revealed YopM in the nuclear fraction and YopE in the cytosolic fraction, thus indicating that YopM was targeted to the host cell nucleus while YopE was localized to the cytosol (Figure 13-9).

A modification of this approach using phosphorylatable peptide tag-based reporters was developed to measure the translocation of effector proteins into cultured eukaryotic host cells. The first reporter system used a peptide tag containing a **nuclear localization signal (NLS)** sequence, which directed the effector protein into the nucleus of the host cell, and a portion of the eukaryotic transcription factor Elk-1 (amino acids 375 to 392) that is recognized and

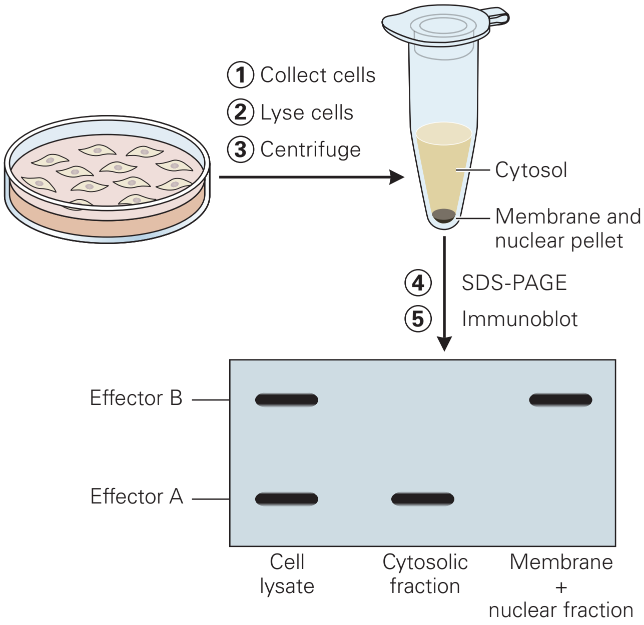

Figure 13-9. Subcellular fractionation and immunoblotting to localize delivered effector proteins. Shown are steps used to separate subcellular compartments of host cells and the expected immunoblot results from subcellular fractionation. Cellular lysates are generated from host cells exposed to a bacteria possessing a T3SS and two effector proteins: one that is delivered into the host cytosol (Effector A) and another that has an NLS, which targets it for the host cell nucleus (Effector B). Antibodies against the two T3SS effector proteins are used for detection of the proteins in the immunoblot. Lysis of the cells through shearing without detergent would yield a supernatant that contains soluble cytosolic components (cytosolic fraction) and a pellet after centrifugation that contains both membranes and nuclei (membrane and nuclear fraction), while treatment with a mild detergent, such as digitonin, to permeabilize the membrane would yield a pellet containing mostly nuclei (nuclear fraction). The different subcellular fractions are then run on SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted using antibodies against the two T3SS effector proteins. The differential localization of the two effectors are reflected in their appearance in the different subcellular fractions.

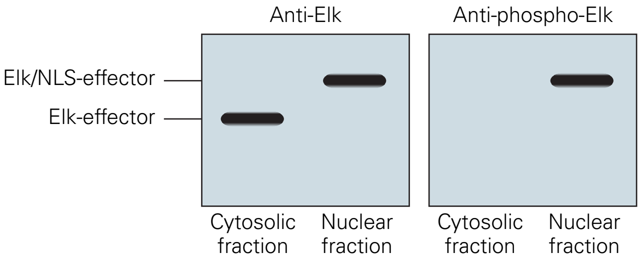

Figure 13-10. Detection of translocated Elk/NLS-tagged effector proteins. Shown are the expected results from SDS-PAGE and the immunoblotting of subcellular cytosolic and nuclear fractions from cellular lysates of host cells exposed to bacteria possessing T3SS and carrying a plasmid encoding an Elk-tagged effector protein either with or without a nuclear localization sequence (NLS). For the left blot, antibodies recognizing the Elk peptide tag were used, whereas for the right blot antibodies specifically recognizing the phosphorylated Elk peptide tag were used. A host kinase located only in the nucleus phosphorylates the Elk peptide tag of the effector protein. Note that the bands for the Elk/NLS-tagged proteins ran higher on the gels than those without the NLS due to the increased size of the tagged protein.

phosphorylated at serine-383 by eukaryotic protein kinases such as Erk2. Translocation of the Elk/NLS-tagged effector protein into the host cell results in transport of the effector protein into the host nucleus, where a host kinase phosphorylates it. Phosphorylation can be detected with antibodies that specifically recognize the phosphorylated peptide sequence on the tagged effector protein (Figure 13-10).

Later, another reporter tag was developed that utilizes a peptide derived from residues 1 to 13 of glycogen synthase kinase 31 (GSK). Translocation of a GSK-tagged effector protein into a eukaryotic cell results in host cell protein kinase-dependent phosphorylation of the GSK tag at serine-9, which can be detected with antibodies. The advantage of this tag is that it does not require an NLS sequence tag because it is phosphorylated by cytosolic protein kinases. This technique has been successfully used to monitor translocation of effector proteins from both the T3SS and T4SS (discussed in the following section).

Researchers have since combined and modified the above approaches by using confocal fluorescence microscopy to observe subcellular localization and/or other reporters, such as green fluorescent protein and β-lactamase, to show the delivery of effector proteins into the host cell. For instance, like the previously mentioned approaches, translocation of a modified effector protein could be measured as a change in fluorescence (Figure 13-11). First, eukaryotic cells are cultured in the presence of a special fluorescence reporter. This dye consists of two fluorophores capable of undergoing **fluorescence resonance energy transfer (FRET)** connected by a β-lactam antibiotic linker with hydrophobic ester modifications of its free hydroxyl groups. The ester modifications allow the fluorescent reporter to pass through the cellular membrane, but once in the cytosol these ester groups are hydrolyzed, trapping the molecule inside the cell cytosol. Due to FRET, so long as the β-lactam antibiotic linker remains intact, the close proximity of the two fluorophores in the reporter means that when the first fluorophore is excited, resonance transfer occurs and the second fluorophore emits light. However, in the presence of β-lactamase enzymes, the reporter will get cleaved, separating the two fluorophores so that excitation of the first fluorophore results in light being emitted directly by that fluorophore without resonance transfer occurring. Basically, the β-lactamase enzyme results in the fluorescent reporter changing color. The translocation assay itself involves fusing β-lactamase to the translocated effector. When this β-lactamase-effector fu-

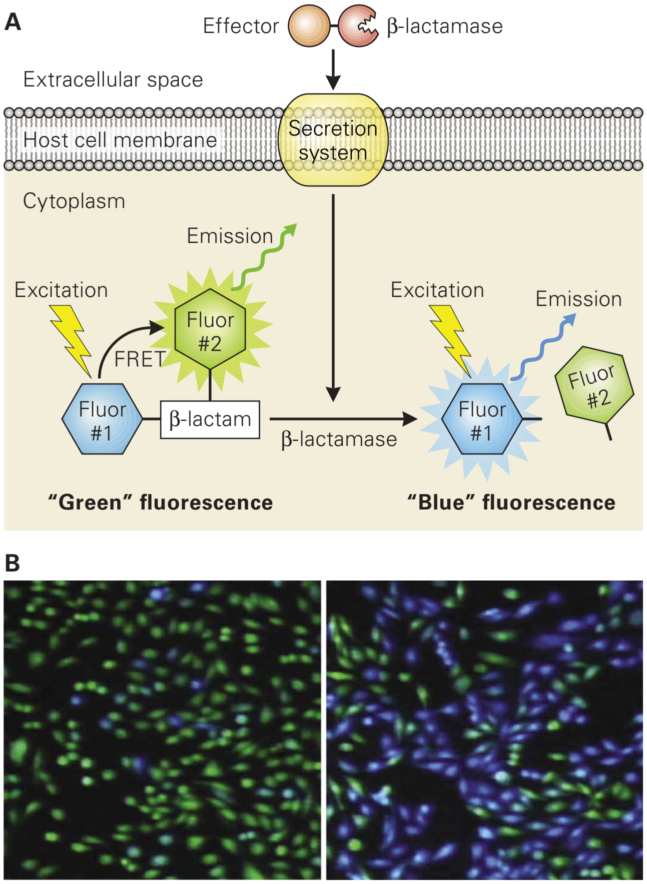

Figure 13-11. Fluorescence resonance energy transfer (FRET) translocation assay. (A) Host cells are loaded with the fluorescence reporter, which has a β-lactam ring linked to the two fluorophores. FRET occurs when excitation of the donor fluorophore (Fluor #1) results in photon energy transfer and emission from a nearby acceptor fluorophore (Fluor #2), resulting here in green fluorescence. When β-lactamase is delivered into the cell along with a secretion system effector, the β-lactamase cleaves the β-lactam ring, allowing the two fluorophores to separate and thus preventing FRET. Now that FRET cannot occur, excitation of Fluor #1 results in it directly emitting blue fluorescence. **(B)** At the cellular level, the host cells go from green (left panel) to blue (right panel), as seen by fluorescence microscopy. Images courtesy of Brenda A. Wilson.

sion is delivered into target cells preloaded with the FRET reporter, the reporter will be cleaved, resulting in a color change detectable using fluorescence microscopy. This technique has been successfully applied to T3SSs, as well as T4SSs and T6SSs (discussed in the following sections).

T4SS. The type 4 secretion system (T4SS) involves an elaborate complex of 12 or more proteins that form a tunnel-like apparatus that directly transfers proteins and/or DNA into host cells in an ATP-dependent manner. There are two main subfamilies of T4SSs. The first subfamily is comprised of conjugation systems that are used to transfer DNA (usually plasmids but also integrative chromosomal elements) and proteins from the **donor** bacterium carrying the conjugative system to another **recipient** bacterium. These systems are also responsible for a significant portion of the **horizontal gene transfer (HGT)** observed in nature. Notably, genes conferring antibiotic resistance are frequently transferred among bacteria through these conjugative systems and will be discussed further in chapter 16, which covers antibiotic resistance. Two notable conjugation systems have been instrumental in the development of modern bacterial genetics: those encoded on the F plasmid and those on the incP plasmid. The DNA transfer properties of the F episome were instrumental in the early genetic mapping of *E. coli*, while the conjugal transfer properties of incP plasmids have formed the basis of our ability to genetically alter numerous bacterial species. Yet perhaps the best-studied of the conjugative T4SSs is the Ti complex (VirB/VirD system) from *Agrobacterium tumefaciens* (a plant pathogen that causes crown gall tumors), which transfers oncogenic Ti plasmid DNA and proteins from the bacterium directly into plant cells, resulting in tumor formation in the plants.

The second major subfamily of T4SSs has been identified in pathogenicity islands (PAIs) in a number of pathogenic bacteria, including: the *cag* system in *Helicobacter pylori* (which causes gastritis, ulcers, and stomach cancer); the Dot/Icm systems in the intracellular pathogens *Legionella pneumophila* and *Coxiella burnetii*; and the VirB/VirD4/Trw systems in the intracellular bacteria *Brucella* (which causes brucellosis), *Bartonella* (which causes cat scratch fever), *Rickettsia* (which causes typhus and spotted fever), *Mesorhizobium loti* (a symbiont of plant cells), and *Wolbachia* (a symbiont of nematodes that causes river blindness). Although the T4SSs in these bacteria are important for virulence and intracellular survival, not all of the effector proteins have been identified. Some T4SS components of the Ptl system in *B. pertussis*, the causative agent of whooping cough, have evolved to secrete pertussis toxin into the medium rather than directly injecting it into the host cell. Even though pertussis toxin subunits have Sec signal sequences and use the general Sec system to be transported to the periplasm, the Ptl system is used to transport the assembled pertussis toxin complex out of the periplasm and into the medium in a contact-independent manner (Figure 13-12B).

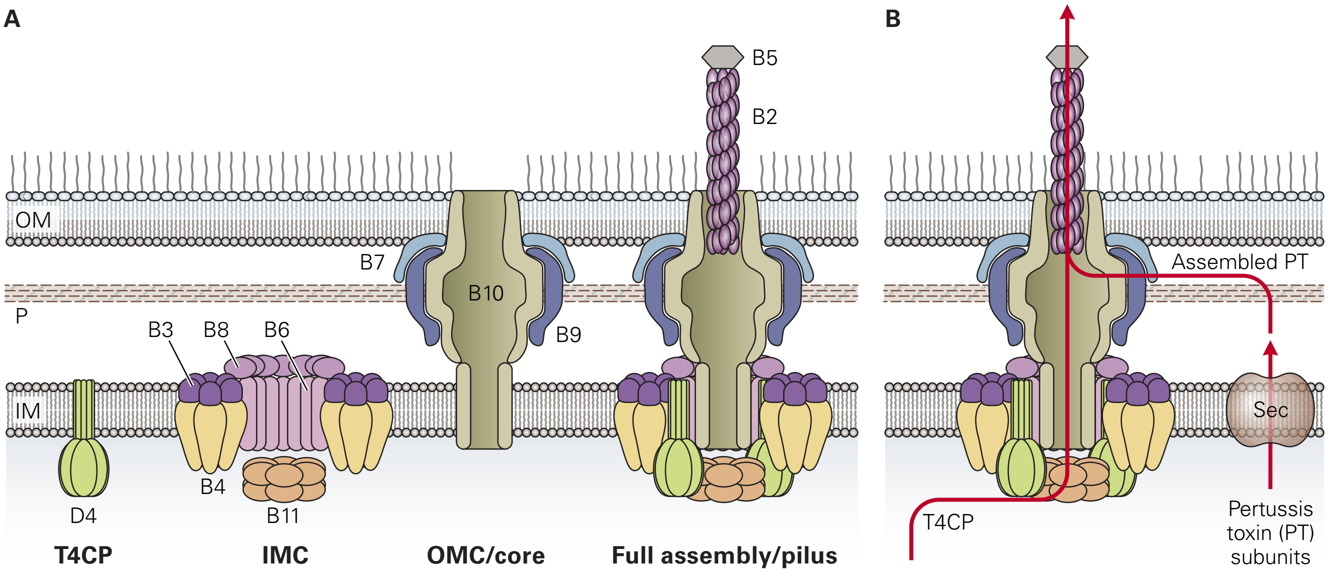

Figure 13-12. The T4SS. (A) Schematic diagram showing the main component complexes of the VirB/VirD4 T4SS found in *Agrobacterium tumefaciens*. The T4SS coupling protein (T4CP) is comprised of VirD4 and assembles in the inner membrane. The inner membrane complex (IMC) is comprised of VirB3, B4, B6, B8, and B11. The outer membrane complex (OMC) is comprised of VirB7 and VirB10 and spans the periplasmic space. The pilus is comprised of VirB2 and VirB5. **(B)** Secretion substrates are first recruited by the T4CP and then fed through the IMC and OMC. VirD4, VirB4, and VirB11 are ATPases that provide the energy required for substrate translocation. In the Ptl system of *B. pertussis*, substrates are first delivered to the periplasmic space through the general Sec secretion pathway before being exported through the T4SS. Adapted from Christie PJ, Whitaker N, González-Rivera C. 2014. *Biochim Biophys Acta* **1843:**1578–1591, with permission.

The T4SS apparatus can generally be thought of as an assembly of four different component complexes (Figure 13-12A). The first is an inner membrane ATPase T4CP (type 4 secretion system coupling protein), which is responsible for bringing substrates to the translocation machinery. The second is a large inner membrane complex that is responsible for transferring substrates across the inner membrane. This complex has the greatest variability between different families of T4SSs, consisting of entirely different sets of proteins. Some of these proteins are actually conserved in the core inner membrane complex of T6SSs as well (see the following section). The third complex is the outer membrane complex that spans the periplasmic space. It is required for passage of substrates through the periplasm and across the outer membrane. The fourth and final complex is the pilus. It is an extracellular organelle that initiates contact with potential target recipients.

T6SS. Although the type 6 secretion system (T6SS) was first identified as a novel secretion system through proteomic studies of the fish pathogen *Edwardsiella tarda* in 2004, its importance was not fully appreciated until it was recognized as a virulence determinant of certain strains of *V. cholerae* and several of its canonical substrates were identified by a screen for virulence against the amoeba *Dictyostelium discoideum*. *Dictyostelium* amoebae were used because their phagocytic activity as they graze on bacteria closely resembles that of macrophages and other immune cells. Within a few years of its initial discovery, T6SS gene clusters would be identified in nearly a quarter of all sequenced Gram-negative genomes, including both pathogens and environmental species.

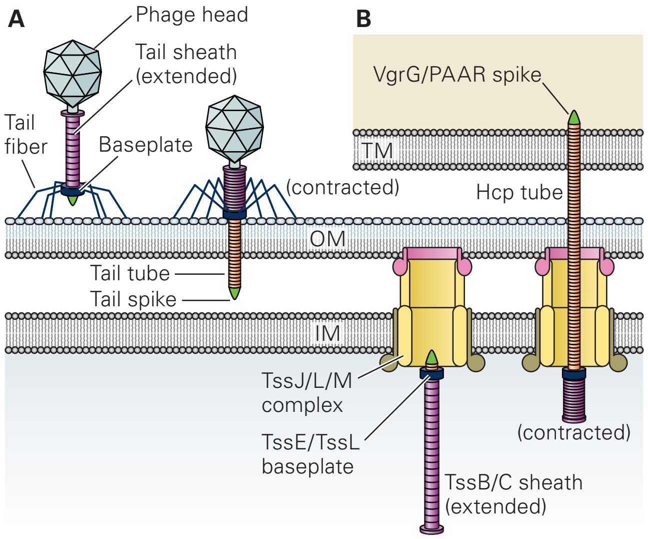

Figure 13-13. Comparison of the T6SS to contractile bacteriophage. The T6SS and contractile phage share a number of key structural components. The phage attaches to the bacterial outer membrane (OM) from the extracellular space, while the T6SS assembles within the inner membrane (IM). Contraction of the phage sheath forces the phage spike into a target bacterial cell, while contraction of the T6SS sheath forces the T6SS spike out of the bacterial cell. If a target membrane (TM) of a neighboring eukaryotic or bacterial cell is close enough, the T6SS spike will cross the TM to deliver effector molecules into the adjacent cell. Protein components conserved in T4 phage and the T6SS are depicted in the same color. Adapted from Ho BT, Dong TG, Mekalanos JJ. 2014. *Cell Host Microbe* **15**:9–21, with permission.

During early bioinformatic analysis of the T6SS, it was quickly noticed that several T6SS components bore a striking resemblance to components of the contractile tail of bacteriophage T4 (Figure 13-13). Indeed, much of our understanding of the T6SS is largely based on our understanding of how contractile-tailed phages work. When a phage encounters a target bacterial cell, its tail fibers and baseplate attach to the target cell and it positions its tail perpendicular to the cell surface. Once positioned, the components of the outer sheath of the phage tail undergo a conformational change resulting in the sheath's rapid contraction. As the sheath contracts, the rigid, spiked inner tube is propelled across the membrane, allowing its genetic payload to be delivered. The T6SS functions analogously, except instead of attaching to the outside of the outer membrane, the T6SS baseplate attaches to an inner membrane protein complex that spans the periplasmic space (Figure 13-14). This means that when the T6SS sheath contracts, its inner tube is propelled out of the cell into the extracellular space and potentially into any target cell nearby.

In the T6SS, the inner membrane complex is comprised of several different proteins, two of which (TssM and TssL) actually have homologs in the inner membrane complex of the Dot/Icm T4SS (IcmF and DotU, respectively). This inner membrane complex spans the periplasmic space and associates with the outer membrane via the lipoprotein TssJ. The T6SS baseplate assembles on the cytoplasmic side of the inner membrane complex and is comprised of at least TssE and TssK, but probably other components as well. The sheath and inner tube are assembled from the baseplate structure. The inner tube is comprised of the protein Hcp. Individual Hcp subunits first form hexameric rings, which then stack on top of each other to form a tube. At the tip of this Hcp tube is a conical spike comprised of a trimer of VgrG proteins. The tip of this VgrG spike is further sharpened by a PAAR (proline-alanine-alanine-arginine) motif-containing protein often simply referred to as PAAR. VgrG and PAAR are interesting because they are frequently found to have C-terminal extension domains that carry various enzymatic activities and were among the first identified T6SS effectors. Additional effectors have been found to associate with VgrG, PAAR, or even the inner lumen of the Hcp rings, suggesting that effector delivery by the T6SS works by delivering the tip of the Hcp tube into target cells. Surrounding the inner tube is the T6SS sheath, which is comprised of two proteins, TssB and TssC (also known as VipA and VipB). At

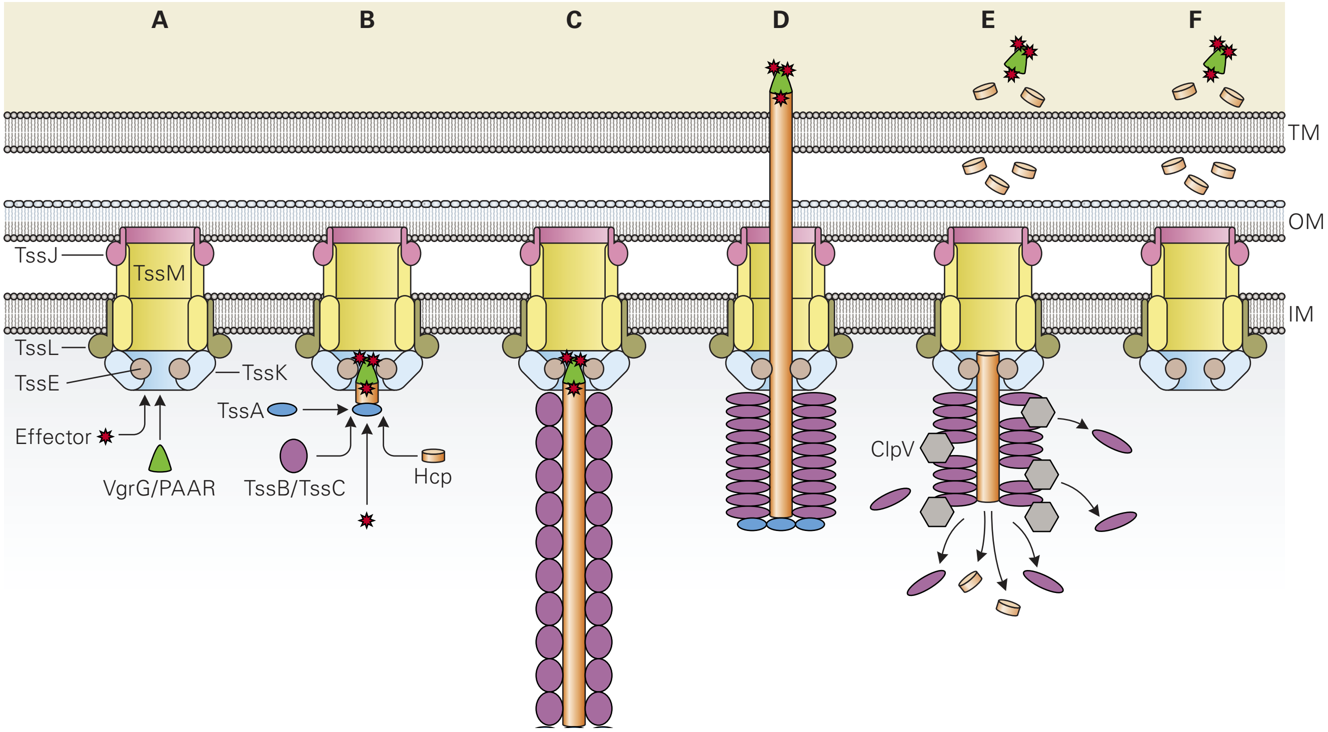

Figure 13-14. Mechanism of T6SS secretion. Shown are the steps involved in assembly of the T6SS core complex. (A) VgrG and PAAR, as well as certain effectors, are recruited to this complex. **(B)** Hcp, sheath components, and other effectors are then recruited so that the tube and sheath can begin to polymerize. VgrG interaction with PAAR or effectors likely contributes to the overall stability of the apparatus assembly. **(C)** The sheath forms around the inner tube in an extended high-energy conformation. TssA forms a cap at the growing end of the tube/sheath assembly. **(D)** Analogous to the phage, sheath subunits undergo a rapid conformational change to a lower-energy form, which results in a contraction that launches the Hcp tube out of the cell and across a target membrane (TM), delivering the loaded VgrG-effector "warhead" into the target cell. **(E)** Through an ATP-driven process, the ClpV protease complex remodels the contracted sheath, restoring the pool of available sheath subunits in their high-energy conformation. The now unsheathed Hcp tube disassembles, and the parts of the tube that are not expelled from the bacterial cell are recycled within the cytosol. **(F)** The naked core complex is then ready to be recycled or disassembled, depending on the T6SS and its activation state. Adapted from Ho BT, Dong TG, Mekalanos JJ. 2014. *Cell Host Microbe* **15**:9–21, with permission.

the opposite end of the sheath/tube assembly from the VgrG spike is a cap complex comprised of subunits of TssA. This complex is required for addition of new Hcp and sheath subunits during assembly.

Despite their similarities, there are several key differences between contractile phages and the T6SS. First, they drastically differ in their size. Although both consist of contractile sheaths and an inner tube, the phage tail is considerably shorter (~100 nm) than the fully assembled T6SS (~1 μm). This difference in length allows bacteria carrying a T6SS to reach target membranes by simply being adjacent to them. As such, targets of T6SSs do not require a binding receptor, as for phage or T3SSs. This allows T6SSs to have a very wide range of potential targets, including both eukaryotic cells and other bacteria.

Second, for phages, once the inner tube is propelled across the target membrane, it serves as a stable translocation channel through which the phage's genetic material enters the cell. However, for T6SSs, the inner tube does not appear to function as a translocation channel, as the tube's integrity is not stably maintained outside of a cell. Rather, effector proteins are loaded onto the tip of the tube either by associating with structural components of the tube through noncovalent protein-protein interactions or through gene fusions to structural components VgrG, PAAR, or Hcp. When the T6SS sheath contracts and the inner tube is propelled out of the cell, and if it can reach a target membrane, the effector-loaded tip of the inner tube is deposited into the target cell, much like a poison-tipped spear being stabbed into an enemy.

Third, phages utilize a so-called one-and-done mechanism in which a single contraction event results in penetration of the target membrane and infection of the target cell. After delivery of the genetic payload, the contractile delivery machinery is discarded. However, because the T6SS machinery is primarily contained within the bacterial cell, its components can be reused. Indeed, secretion by the T6SS is a dynamic process involving cycles of sheath/tube assembly, followed by contraction and secretion, and concluding with disassembly of the contracted sheath. Recycling of the sheath subunits is mediated by the ATPase ClpV and provides the energy required for secretion. When individual sheath subunits assemble, they do so in a high-energy conformation, analogous to a stretched-out rubber band. Sheath contraction is simply the release of this stored-up energy. In order to restore energy to the system, ClpV consumes ATP to unfold and disassemble the contracted sheath subunits.

Several T6SSs have been demonstrated to contribute to the virulence of several known pathogens, most notably in certain strains of *Vibrio, Aeromonas, Pseudomonas, Burkholderia,* and *Salmonella* species. However, what makes the T6SS stand out from other secretion systems is that it is responsible for mediating interbacterial competition. Even T6SSs with clear eukaryotic-specific toxin effectors, such as those found in *Vibrio* and *Aeromonas*, also secrete T6SS effectors that very efficiently destroy other bacteria. The antibacterial T6SS effectors from different species include various types of nucleases, lipases, and lysozymes. In general, all of these antibacterial effectors are accompanied by a cognate immunity protein that prevents self-intoxication or antagonism between sister cells of the same strain.

Some T6SSs, such as those found in several different *Pseudomonas* species, have even evolved elaborate regulatory systems that allow it to selectively target antagonistic neighbors while leaving nonthreatening neighbors unharmed. When a *Pseudomonas* cell is attacked by the T6SS of an adjacent bacterium, it will assemble a T6SS apparatus precisely at the point of attack and launch an aimed retaliatory counterattack at the initial aggressor. Furthermore, the *Pseudomonas* T6SS not only recognizes exogenous T6SS attacks but also other forms of membrane perturbing attacks, such as T4SS-mediated conjugation and certain membrane-permeabilizing antibiotics. Ultimately, the T6SS plays an important role in maintaining the balance between different bacteria in multispecies mixtures, such as those found within the human gut. Exactly what role it plays in human health and disease is an exciting area of active research.

Specialized Secretion Systems Specific to Gram-Positive Bacteria

Gram-positive bacteria lack an outer membrane, so proteins exported by the Sec and Tat translocation systems appear directly on the cell surface or in the medium. This arrangement causes several problems not encountered by Gram-negative bacteria. Foremost among these is that the secreted proteins intended for cell surface localization must interact strongly with cell surface components to prevent secretion and release into the surrounding medium. Some of these proteins are covalently linked to the peptidoglycan by the sortase system (see chapter 11). Other proteins contain transmembrane anchors that insert into the outer membrane or that are attached to membrane lipids and become lipoproteins attached to the cell surface. Still other proteins bind to the peptidoglycan or teichoic acids on the surface through binding domains such as LysM or CBP (choline-binding protein), respectively. Finally, some proteins form ionic or hydrophobic contacts to specific proteins embedded in the cell membrane or to the membranes themselves.

General secretory protein export systems in Gram-positive pathogens are arranged into several different organizations, which likely optimize their surface structures for maximal growth and possibly toxin export and delivery. As noted earlier, there are special accessory Sec secretion systems in some Gram-positive pathogens for export of glycosylated toxins. In addition, *S. pyogenes* (Group A strep) has an unusual mechanism for delivery of a specific toxin into the cytoplasm of host cells. Finally, some Gram-positive bacteria, such as *Mycobacterium*, have unusual waxy cell walls that require a special export system, called the type 7 secretion system (T7SS). The other major specialized protein exporters discussed previously for Gram-negative bacteria, such as T3SSs and T4SSs, are absent from Gram-positive bacteria. However, versions of T4SSs are still expressed and utilized in Gram-positive bacteria for DNA exchange processes, such as conjugation, which transfers plasmids and chromosomal DNA between cells and is important for horizontal gene transfer (see chapter 7).

General Secretory Transporter Systems in Gram-Positive Bacteria

Gram-positive bacteria organize their SecA-SecYEG transporters into several different patterns. In *Enterococcus faecalis*, the SecA-SecYEG transporter locates to a single focus at the equators of growing cells before they are about to divide, but becomes a septal band around the equator (part of the cell division septum) at later stages of cell division. The sortase enzyme (SrtA) that covalently attaches secreted proteins to the peptidoglycan (see chapter 11) colocalizes with the SecA-SecYEG transporter, thereby providing a mechanism for efficient attachment of exported virulence factors to the cell surface of these bacteria. In *S. pyogenes* (Group A strep), SecA has also been reported to localize at a single focus or microdomain, termed the **ExPortal**, in the late exponential growth phase, although some studies indicate that SecA is uniformly distributed around the cell periphery in exponentially growing and stationary-phase *S. pyogenes* cells. The ExPortal has been proposed to function as an organelle that coordinates interactions between some proteins secreted by the Sec system and membrane-associated chaperones or sortases. This type of coordinated secretion may be important in Gram-positive bacteria without a periplasmic space where folding of Sec-secreted proteins usually occurs.

For instance, the SrtA sortase of *S. pyogenes* localizes to distinct loci during the cell cycle, possibly with the ExPortal microdomain. Microdomain colocalization of the SecA-SecYEG transporter and SrtA sortase have been reported for the oral pathogen *Streptococcus mutans*, and SecA and SrtA colocalize at the equatorial rings and division septa of *Streptococcus agalactiae* (Group B strep). How sortase-attached proteins distribute over the surfaces and poles of cells is not well understood in these bacteria with highly localized SrtA sortases. In contrast, the SecA-SecYEG transporter of the respiratory pathogen *Streptococcus pneumoniae* (pneumococcus) localizes dynamically over the mid-cell region of actively dividing cells, whereas the SrtA sortase distributes uniformly over the periphery of cells. Interestingly, an extracellular protease involved in degrading misfolded proteins localizes with the SecA-SecYEG transporter at mid-cell regions, possibly optimizing quality control of exported proteins. The SecA-SecYEG transporter has been reported to localize in a spiral pattern over the surface of rod-shaped Gram-positive bacteria, such as *Bacillus subtilis.* In this case, the Sec translocation system seems to be specifically organized over the whole length of the bacterial cells.

Taken together, these different patterns of SecA-SecYEG transporter organization likely optimize protein export for different physiological needs of these bacteria. For example, peptidoglycan biosynthesis involves a large number of bitopic membrane proteins, which are partly embedded in the membrane by the SecA-SecYEG transporter (see earlier discussion). Therefore, SecA-SecYEG transporters and quality-control proteases may need to be concentrated to regions of active cell wall biosynthesis. On the other hand, *S. pyogenes* (Group A strep) and certain other bacterial pathogens secrete a relatively large number of toxins to host cells, compared to other bacteria such as *S. pneumoniae,* suggesting the possible need for a dedicated ExPortal microdomain at certain stages of infection. Considerably more work needs to be done in Gram-positive pathogens to understand the dynamics, localization, and regulation of general protein secretory transporter systems.

Cytolysin-Mediated Translocation (CMT) in *S. pyogenes* (Group A Strep)

Toxin-rich *Streptococcus pyogenes* has evolved a mechanism to deliver a specific excreted toxin to adjacent host cells. Through a process termed **cytolysin-mediated translocation (CMT)**, *S. pyogenes* utilizes a pore-forming toxin to enable delivery of an effector protein secreted by the general Sec system directly into the eukaryotic cells. CMT in *S. pyogenes* is mediated by the pore-forming toxin **streptolysin O (SLO)**, a member of the cholesterol-dependent cytolysin class of toxins that bind to cholesterol molecules in host cell membranes, thereby leading to pore formation that kills host cells. However, SLO has evolved a second cholesterol-independent mode of interaction with host membranes that allows it to interact with the effector protein *S. pyogenes* β-NAD$^+$ glycohydrolase (SPN), which cleaves the cofactor β-NAD$^+$ in the cytoplasm of host cells.

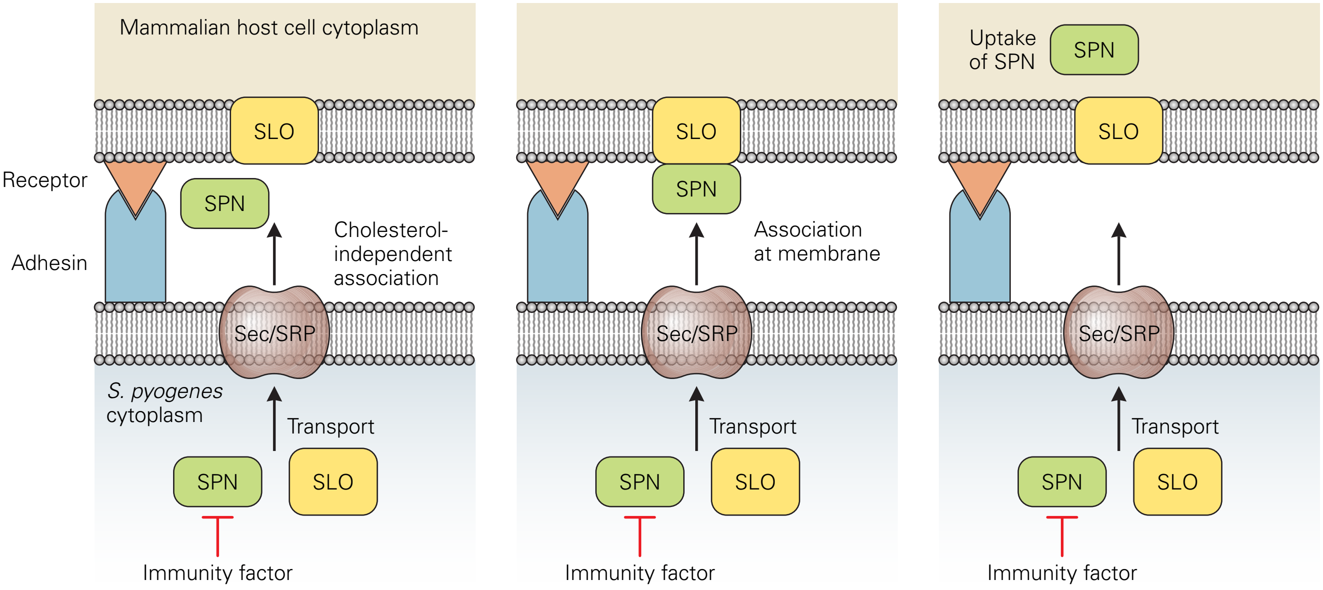

Figure 13-15. Model for uptake of SPN toxin into host cells by cytolysin (SLO)-mediated translocation (CMT) in *S. pyogenes* (Group A strep). Streptolysin O (SLO) is a cholesterol-dependent cytolysin (CDC) that can form pores in and puncture host cell membranes. However, in CMT, the extracellularly secreted SLO functions in a cholesterol-independent way to interact with host membranes, possibly via an unknown receptor protein, and with secreted SPN toxin. This mechanism requires that expression and secretion of SLO and SPN are tightly coregulated. SPN is then taken up into the host cell cytoplasm, possibly via vesicle formation, while SLO remains associated with the host membrane. SPN is an enzyme toxin that breaks down the essential cofactor β-NAD$^+$ in the host cell cytoplasm. The bacteria protects against SPN toxin activity before secretion by producing an immunity factor that inhibits the SPN enzyme and by keeping SPN unfolded, possibly through cotranslational secretion by the SRP system. To date, CMT has been found only for this specific pair of toxins produced by *S. pyogenes* (Group A strep). Data from Madden JC, Ruiz N, Caparon M. 2001. *Cell* **104:**143–152.

According to the current model (Figure 13-15), when SLO interacts at host membranes in the presence of SPN, the cholesterol-binding mode of SLO pore formation is suppressed and pores are not formed. The SLO-SPN complex may interact with an as-yet unidentified receptor in the host membrane. Following association with the host membrane through binding of an adhesin, the SPN toxin is rapidly internalized into the host cell cytosol. Notably, SLO and SPN are encoded by genes in the same tightly controlled operon, and both proteins are secreted at least partly by the signal-recognition particle (SRP) mechanism of protein export discussed previously. In fact, it seems that only SLO and SPN produced by the same bacterium mediate CMT of SPN into a given host cell, suggesting a requirement for close adhesion between bacteria and host cells. Both the SLO and SPN proteins have extra amino-terminal domains required for CMT, and the SLO cytolysin has evolved additional amino acid changes so that it can function in a cholesterol-dependent way to form pores or in a cholesterol-independent way to mediate CMT. The transport of SPN by CMT is highly specialized and specific to *S. pyogenes.* Whether CMT is a delivery system common to other Gram-positive bacteria still remains to be determined. On the other hand, an increasing number of examples of binding of different cytolysins to receptor proteins in host cell membranes have been reported, suggesting that other cytolysins may have additional roles in virulence processes besides pore formation.

Type 7 Secretion System (T7SS)

Gram-positive mycobacteria with high GC content, such as *M. tuberculosis*, the causative agent of tuberculosis, have highly unusual surfaces called mycomembranes. This barrier consists of hydrophobic mycolic acids that are covalently attached to the peptidoglycan and various free lipids that are embedded in the mycolic acids. An exopolysaccharide capsule layer further covers the mycomembrane. The mycomembrane makes DNA uptake and cell staining difficult, yet mycobacteria are still able to secrete proteins into their growth media. Two secreted proteins that are important T cell-antigenic targets required for virulence, now known as EsxA and EsxB (formerly referred to as ESAT-6 and CFP-10, respectively), are pore-forming toxins involved in escape from macrophage phagosomes. Comparative genomics of the pathogenic *M. tuberculosis* genome with attenuated *Mycobacterium* vaccine strains revealed that the genetic region surrounding the genes encoding EsxA and EsxB was present only in the pathogenic strain. Interestingly, during complementation studies in which the genes encoding these secreted proteins were reintroduced, their expression, not secretion, was restored. Only when the entire missing genetic locus was reintroduced did secretion occur, indicating that this genetic locus encoded a protein secretion system. Because the genes in this locus were different from the previously known secretions systems, it was given its own designation, the **type 7 secretion system (T7SS)**.

Mycobacteria can have up to five different T7SSs, ESX-1 through ESX-5. These T7SSs apparently arose through gene duplication events from the original ESX-3 T7SS. The mechanism of the T7SS is not fully understood, but some features have been worked out, particularly from studies of the EXS-1 system (Figure 13-16). EsxA and EsxB form a heterodimer and are secreted in a folded conformation. At the C-terminus of EsxB, there is a flexible tail that carries a motif required for secretion. Indeed, this motif has been

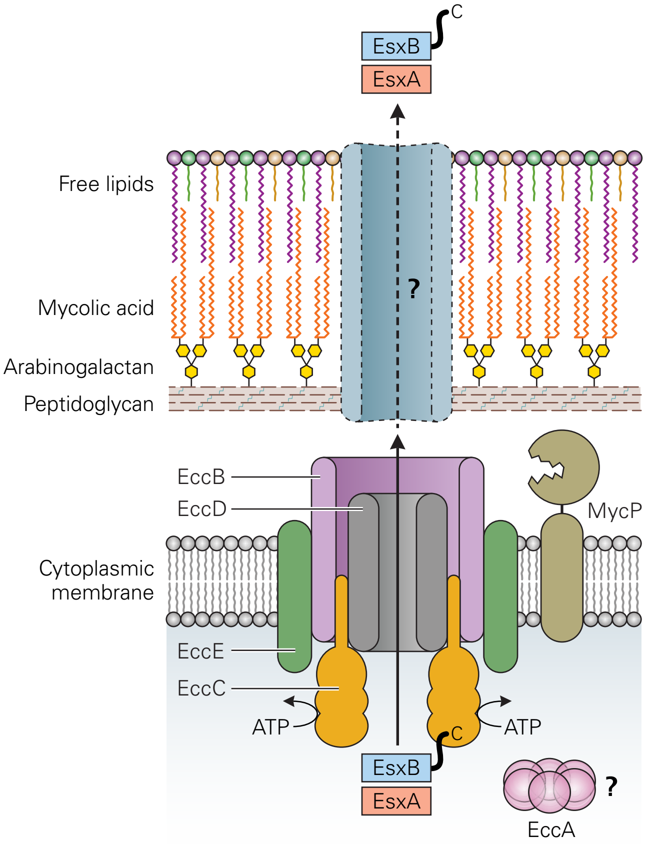

Figure 13-16. The T7SS. Shown here is the inner membrane complex of the ESX-1 T7SS found in *Mycobacteria*, which consists of EccB, EccC, EccD, and EccE. Substrates like EsxA and EsxB (also called ESAT-6 and CFP-10, respectively) have a C-terminal secretion signal that is recognized by EccC. EsxA is a pore-forming toxin that helps the *Mycobacteria* escape from the macrophage phagosome. EccB is a hexameric ATPase that helps drive the transport of effectors. MycP is a mycosin protease that cleaves the EsxB substrate and thereby induces a heptameric oligomerization involved in pore formation within phagosomes. MycP and the multimeric EccA are associated with the T7SS, but their roles remain unclear. Once substrates are secreted into the periplasmic space, how they are able to cross the outer mycomembrane also remains unknown. Based on information from Costa TR, Felisberto-Rodrigues C, Meir A, Prevost MS, Redzej A, Trokter M, Waksman G. 2015. *Nat Rev Microbiol* **13:**343–359.

identified in all known mycobacterial T7SS substrates or substrate complexes and, as such, can be considered to be a general secretion signal. This signal of EsxB has been shown to bind to a conserved inner membrane protein, EccC. EccC has significant homology to a large family of ATPases involved in various cellular processes and forms a hexameric structure. The best-known members of this family are FtsK and SpoIIIE, which actively transport DNA into daughter cells during cell division and sporulation, respectively. Another member of this family is T4CP, the coupling protein of the T4SS discussed earlier in this chapter. All three of these family members are involved in the active transport of substrates across the cellular membrane, suggesting that EccC plays a similar role in the T7SS. EccC forms a complex with other inner membrane components EccB, EccD, EccE, and MycP, and together they likely form the pore complex through which substrates leave the cytoplasm, although this has not yet been confirmed. Once substrates leave the cytoplasm, it is still unclear how they cross the outer mycomembrane, but MycP appears to be a protease that cleaves the ExsB protein to induce its oligomerization. Additionally, another multimeric ATPase, EccA, is commonly found to be associated with the T7SS, but its precise role remains unknown.

Although the T7SS has been mostly studied in *Mycobacteria*, T7SS gene clusters are also present and functional in several other species of Gram-positive pathogens, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Streptococcus agalactiae* (Group B strep), and *Bacillus anthracis*. It is not clear why species lacking a mycomembrane would also make use of the T7SS, but one possibility is that the T7SS may play additional roles beyond simple secretion of protein substrates. In fact, in *M. smegmatis,* a nonpathogenic relative of *M. tuberculosis*, the T7SS is actually involved in a special form of conjugation in which different large chromosomal DNA fragments can be exchanged between certain donor and recipient strains. Understanding both the mechanistic details and potential purposes of the T7SS in these different species are areas of active research.

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Questions

1. *Shigella* expresses a number of virulence factors. Describe in detail how you would show which of the *Shigella*-secreted proteins are actually translocated into host cells during infection.

2. Describe how you would show that putative translocated effector proteins from *Salmonella* were involved in inducing actin rearrangements, changing protein phosphorylation states of host proteins, and causing induction of apoptosis or DNA synthesis in host cells.

3. Why do secretion systems differ so much in their strategies? Why don't all of the systems directly inject proteins into mammalian cells? In other words, what are the pros and cons of the different secretion systems for the pathogen?

4. In the chapter, we mention that one advantage of direct delivery of toxic effectors into target host cells by the T3SS, T4SS, or T6SS is that the toxic effectors are able to avoid being neutralized by circulating antibodies. What other advantages does direct delivery of a toxic effector have for the pathogen?

5. How could a secretion system like some T4SSs secrete DNA when these systems mostly secrete proteins? (Hint: the first step in conjugation covalently attaches a protein to one strand of the nicked DNA.) How could you show that this explanation of DNA transfer by a protein secretion system is correct?

6. You suspect that a newly discovered secretion system is important for virulence. How would you test this hypothesis? Keep in mind that mutations that disrupt an essential secretion system may kill the cell, thus making the bacterium appear less virulent.

7. Could you use a secretion system to secrete a protein it does not normally secrete? This has been considered in some biotechnology applications in order to avoid having to lyse bacterial cells. What type of secretion system would you use in the case of a Gram-negative bacterium, or is there one? What about a Gram-positive bacterium? What would you have to do in the way of genetic engineering to make this work? Why might your strategy fail?

8. You want to design a plasmid vector that would enable researchers to have their favorite protein(s) secreted by *E. coli*, even though that protein is not normally a secreted protein. Which of the secretion systems would you choose and why? Describe what this plasmid vector would entail.

9. What are the different functions of cytoplasmic and periplasmic chaperones in secretion?

10. How does the autotransporter secretory pathway differ from the other secretory pathways? Is this secretion mechanism considered Sec-dependent or Sec-independent?

11. Why do Gram-positive bacteria lack many of the specialized protein export systems found in Gram-negative bacteria? What possible disadvantages does a lack of these export systems impart to Gram-positive pathogens? Are there any advantages for Gram-positive bacteria in not producing these export systems?

12. Describe the mechanisms used by Gram-positive bacteria to retain proteins on their cell surfaces. Why are these mechanisms needed?

Solving Problems in Bacterial Pathogenesis

1. Although most community-acquired methicillin-resistant *S. aureus* (CA-MRSA) bacteria are not as resistant to antibiotics as the hospital-acquired MRSA strains, the recently identified strains that we hear about in the news appear to be more virulent. A group of researchers recently discovered that bacterial cultures of a particularly virulent strain of CA-MRSA (called USA300) cause apoptosis in leukocytes and neutrophils, as evidenced by release of cytochrome c from mitochondria, followed by cytokine release and cell death. Antibodies generated against a bacterial culture medium from the USA300 strain identified two proteins of 33 kDa and 44 kDa on Western blots. To determine the localization of these two proteins, researchers performed sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot analysis of lysates from the bacterial cell pellets and the bacterial culture medium, as well as lysates of human neutrophils treated with the USA300 strain. The results from the Western blot analysis are shown in the left blot in Figure 1. The researchers then applied the cell-free bacterial culture medium to the neutrophils and performed subcellular fractionation of the treated neutrophils. The results from the Western blot analysis are shown in the right blot in Figure 1.
   a. Provide an interpretation (with rationale) of the results shown in Figure 1.
   b. Predict (with rationale) possible functions for the two proteins, which have been named LukS and LukF (LukS is the 33-kDa protein and LukF is the 44-kDa protein). Provide at least one additional (different) experiment that could be performed to confirm your prediction.
   c. The researchers speculate that the two proteins, LukS and LukF, are two subunits of an AB toxin. Based on the results shown in Figure 1, do you agree with this interpretation? Provide your rationale. Provide an experiment that would allow you to verify your answer.

2. A group of researchers at the university veterinary diagnostics laboratory isolated a new bacterium from the blood and stool of several horses that became ill and died at a local farm. Prior to death, symptoms included disorientation, loss of motor function, and flaccid paralysis, so the researchers suspected central nervous system involvement and possible production of neurotoxin. Based on 16S rRNA comparison, they found that the new bacterium was related to the Gram-positive bacterium *Clostridium botulinum*, and they named it *Clostridium equiniae*. The researchers subsequently developed a mouse model of infection, in which bacteria are injected into the bloodstreams of mice and the mice are monitored for paralysis and death. Using this animal model, they have conducted transposon mutagenesis to identify genes involved in virulence. From these studies, they have isolated three avirulent mutants (CeMut1 through CeMut3) in which genes that encode putative virulence factors have been deleted. They have conducted a series of experiments to determine the role of each of these virulence factors in pathogenesis. Interestingly, the researchers subsequently found that CeMut1, while avirulent, still produces and secretes the proteins encoded by the genes deleted in CeMut2 and CeMut3. Sequence comparison with protein databases revealed that the gene deleted in CeMut2 encodes a protein of 50 kDa and has significant sequence similarities to the catalytic N-terminal domain of botulinum neurotoxin (therefore, they named this protein BltA for botulinum-like toxin A part). The gene deleted in CeMut3 encodes a protein of 100 kDa and has significant sequence similarity in its N terminus to cholesterol-dependent cytolysins and in its C terminus to the B subunit of cholera toxin (therefore, they named this protein CptB for cytolysin plus toxin B part). Based on this, they suspected that the two proteins BltA and CptB form an AB neurotoxin and decided to test this idea. They made antibodies against the culture filtrate from wild-type *C. equiniae* and then used the antisera in Western blots to compare the localization of the putative

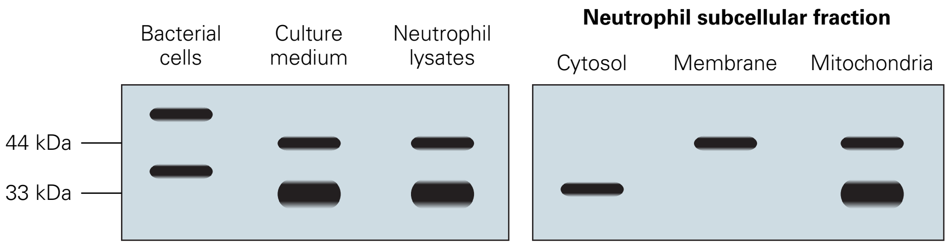

Figure 1. **Western blots using antibodies against USA300-secreted proteins.**

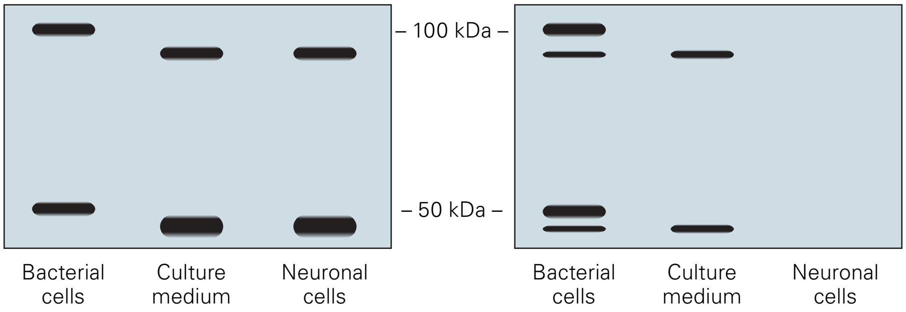

Figure 2. **Western blot of cellular fractions from the wild type (left) and CeMut1 (right) using polyclonal antibodies against secreted proteins from *C. equiniae*.**

toxin proteins in the wild-type and CeMut1 bacteria and the neuronal cells treated with each bacterium. The results are shown in Figure 2.

a. Provide a detailed interpretation (with rationale) of the results shown in Figure 2. Were the researchers correct in their hypothesis? Provide your rationale.

b. Provide (with rationale) a possible function for the protein encoded by the gene deleted in CeMut1. Provide an experiment that could be performed to confirm your prediction.

c. Draw a clearly labeled schematic diagram depicting a possible model that accounts for all of the experimental observations (i.e., show how BltA and CptB are delivered to host cells and show the mode of action of the putative toxin on neuronal cells). Be sure your model is consistent with your answers to parts A and B.

3. Aphids (plant-eating insects) possess several types of symbiotic bacteria. Pea aphids often harbor, as a symbiont, *Hamiltonella defensa*, which is a Gram-negative bacterium related to *Salmonella*. It is believed that the presence of *H. defensa* confers substantial resistance to parasitic wasps, which lay their eggs inside the aphids and are the aphid's natural enemies. In aphids harboring *H. defensa*, the wasp larvae die prematurely, allowing the aphid hosts to develop into adults and reproduce. Recent genome-sequencing data have revealed that *H. defensa* possesses two PAIs that are prophages, each encoding a T3SS and carrying putative virulence genes. The first PAI, called APSE-1, encodes a homolog of the catalytic subunit of Shiga toxin (Stx), produced by *Shigella*, which inhibits protein synthesis by disrupting 28S rRNA. The second PAI, called APSE-2, encodes a homolog of the catalytic subunit of the cytolethal distending toxin (CdtB) produced by *Campylobacter jejuni*, which is a DNase with an NLS that targets it to the eukaryotic nucleus, where it interrupts cell cycle progression by arresting the cells in G2 phase. It is possible that these APSE-encoded toxins might be selectively targeted to destroy larval cells of attacking parasitic wasps. However, neither of these PAIs include genes that might encode the corresponding binding subunits of the two putative toxins, suggesting that the Stx and CdtB proteins might be delivered to the wasp cells by the T3SS. To further characterize this fascinating symbiotic relationship, you have developed a system for genetically manipulating *H. defensa* that is based on *Salmonella* genetic tools, as well as a system for growing wasp larval cells in tissue culture.

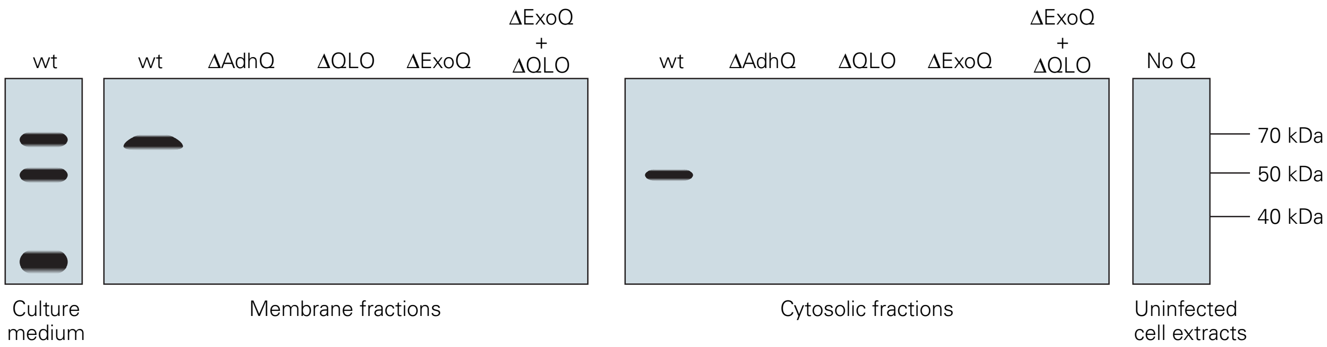

Figure 3. **Western blots of cellular fractions and culture media, using antisera against QLO and ExoQ.**

a. Describe how you would experimentally demonstrate that Stx and CdtB are indeed delivered by the *H. defensa* T3SS into the wasp cells and how they end up in the expected cellular locations. (Hint: you may need more than one type of experiment to demonstrate this.)

b. Describe how you would determine whether these proteins are catalytic A subunits of an AB toxin or T3SS effector proteins.

4. A group of researchers has been studying the pathogenesis of a bacterium Q that they isolated from a patient with a severe sore throat. During the course of their studies, they found that rabbit polyclonal antisera generated against filtered, dialyzed, and concentrated culture filtrates from bacterium Q are reactive against two proteins produced by the bacterium: a 70-kDa protein that has hemolysin-like activity, which they call Q bacteriolysin O (QLO), and a 50-kDa protein that has ADP-ribosyltransferase activity, which they call exotoxin Q (ExoQ). N-terminal sequencing of each protein and comparison with the protein databases allowed the researchers to identify highly homologous genes in several streptococcal species. Using the streptococcal gene sequence information, the researchers cloned the genes from bacterium Q. From sequence analysis, they determined that both proteins are probably synthesized with an additional 25 amino acid residues at the N terminus that are not present in the proteins found in the culture filtrates. The researchers also created in-frame deletion mutants of each gene, which they denoted as ΔQLO and ΔExoQ. They also have a mutant strain of bacterium Q that is deficient in a protein adhesin, denoted ΔAdhQ. All three mutant strains were avirulent in an animal model of infection. The researchers then performed cytotoxicity assays that they developed to examine the effects of the bacteria on epithelial cell cultures. Following exposure of the epithelial cell cultures to the wild-type bacterium and each of the mutants, either alone or as a coinfection (ΔQLO plus ΔExoQ), the researchers washed the epithelial cells to remove the unattached bacteria and lysed the cells. They centrifuged the lysed-cell extracts to separate them into a soluble cytosolic fraction and a membrane pellet fraction. They resuspended the membrane pellet in a buffer containing a mild detergent that solubilizes membranes and proteins but does not lyse bacteria (so that they could then remove the bacteria by centrifugation). They separated the proteins from each of the samples by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred the proteins to a membrane, and detected the proteins by performing a Western blot analysis using the antisera against QLO and ExoQ. The Western blots are shown in Figure 3.

   Using only the information provided and your knowledge of the virulence mechanisms that we covered in this chapter, address each of the following:

   a. Provide an explanation (with rationale) for the difference between the predicted sizes of the QLO and ExoQ proteins and the sizes of the proteins found in the culture medium. How does this occur? Be specific!

   b. Provide a detailed explanation (with rationale) and interpretation for the results shown in the Western blots.

   c. Provide a diagram depicting a possible model that accounts for the experimental observations and your interpretation of them. Be consistent with your answers to A and B. Be sure to clearly label your diagram.

5. By performing mutagenesis studies, a group of researchers have identified a gene (*expA*) that encodes a protein (ExpA) that is involved in degrading extracellular-matrix components. Based on sequence analysis, they suspect that ExpA is secreted via the general secretory pathway.

   a. What feature of the protein sequence led the researchers to this conclusion?

   b. Describe how the researchers might experimentally demonstrate that ExpA is indeed secreted via the general secretory pathway.

   c. What cytoplasmic accessory proteins are important for secreting ExpA?

   d. What function might ExpA have in pathogenesis?

6. You are a graduate student and have performed a transposon mutagenesis screen as part of your dissertation research on a medically important bacterium, *Helpmium graduatii*. Based on previous experimental evidence, this pathogen is thought to deliver at least one cytotoxic effector protein, called HgtA, directly into host cells, presumably via a T3SS. You have identified three independent transposon mutations. You are interested in studying the proteins that are disrupted in these mutants for your doctoral research. You design a series of experiments to characterize these mutants. Using the results for each mutant provided below, answer the questions for that particular mutant.

Mutant 1

- In mutant 1, the effector protein HgtA is constitutively secreted into the supernatant, whereas in the wild-type bacterium HgtA is secreted only in the presence of host cells.
- When you tag the wild-type protein that is mutated with a fluorescent label and look at the wild-type bacterium under the microscope, the protein appears to

be localized on the outside of the bacterial cytoplasmic membrane but remains associated with the cell envelope.

- Overexpression of the protein reduces, but does not inhibit, secretion of HgtA in the presence of host cells.
  a. Is *H. graduatii* a Gram-negative or a Gram-positive bacterium?
  b. Is the protein mutated in mutant 1 a member of a Sec-dependent or Sec-independent pathway?
  c. Propose a function for your protein and provide your rationale.
  d. Why would this function be important to the bacterium?

Mutant 2

- Genetic analysis reveals that the transposon inserted into the first gene of a two-gene operon that encodes two proteins involved in the T3SS.
- Polyclonal antibodies that bind to the two proteins in the wild-type bacterium do not recognize any proteins in the mutant bacterium. In the mutant bacterium, the HgtA effector protein is secreted but cannot be translocated into cultured host cells.
- Sequence analysis of the proteins reveals the presence of at least one region of hydrophobicity in each of the proteins.
  a. The transposon insertion resulted in what type of mutation? How do you know this?
  b. Propose a function for the two proteins in the operon and provide your rationale.

Mutant 3

- Genetic analysis of the mutant bacterium reveals that the transposon inserted into a gene that is adjacent to the gene encoding the HgtA effector protein.
- In the mutant bacterium, secretion of HgtA is severely reduced.
- A polyclonal antibody that binds to wild-type protein (that is encoded by the gene deleted in the mutant 3) detects the protein in the cytoplasm of the wild-type bacterial cells but not in the mutant 3 bacteria.
- The half-life of HgtA is longer in the wild-type bacterium than in the mutant bacterium.
- In the wild-type bacterium, antibodies that bind to HgtA only co-immunoprecipitate the protein encoded by the gene deleted in mutant 3. However, in the mutant bacterium, antibodies that bind HgtA also co-immunoprecipitate a number of other cytoplasmic proteins.
  a. Propose a function for the protein.
  b. Why is this protein important for secretion of HgtA?
  c. Is the function of this particular protein unique? Explain.

14

IN THIS CHAPTER

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CHAPTER 14

Virulence Regulation

Whenever there is a crime spree, law enforcement can become highly motivated to both catch the suspects and be on the lookout for additional would-be criminals. As such, it is often in the best interest of criminal perpetrators to keep their illicit actions in check. By self-limiting their own criminal behavior, they can lay low and stay under the radar of police until their next big heist. Like these elusive criminals, bacterial pathogens have also evolved similar strategies to control their disease-causing potential as they wait for the right opportunities to activate their virulence mechanisms.

Virulence Gene Regulation

Pathogens encounter vastly different environmental conditions, both inside and outside the host. Moreover, once inside the host, pathogens are further exposed to several distinct niches. In each new environment, pathogens are subjected to different stresses, all requiring appropriate responses for the bacteria to survive and grow. Thus, a major step toward understanding how pathogens can cause disease is asking how pathogens transition between these disparate conditions. In other words, how do bacteria that normally reside as saprophytes in vegetative, aqueous, or soil habitats transition to life inside an animal, or in the case of intracellular pathogens, inside a host cell? How can a single extracellular pathogen reside in the very different environments of the nasopharynx, lungs, blood, and brain? To meet these challenges, bacteria have evolved the ability to sense environmental signals, and in response, induce or repress specific sets of genes.

In this chapter, we explore some of the mechanisms by which bacteria switch from one state to another, changing their phenotype to suit their environment or to avoid host defenses, often in coordination with regulation of their virulence genes. We also discuss how bacteria sense signals from their environment and translate that sensory input into modulation of gene transcription and protein expression and function.

Mechanisms of Regulation

The environmental conditions that a pathogen faces during its life cycle, both inside and outside the host, determine the extent to which its virulence factors are regulated. Common factors that influence the expression of virulence-associated genes in bacterial pathogens are: temperature, pH, the abundance of iron (Fe^{2+}) and other divalent metal cations (Ca^{2+}, Mg^{2+}, Mn^{2+}), availability of carbon and nitrogen sources (sugars, amino acids), small molecule signaling compounds, osmolarity, oxygen, carbon dioxide, and light. Genes regulated in response to the host environment that result in a pathogen's ability to survive within that host or to cause disease are referred to as virulence genes. Typically, for any given environmental condition, multiple genes are regulated together. These genes can be regulated either positively or negatively and either concomitantly or sequentially. In this section, we will discuss several of the mechanisms by which this regulation occurs.

Operons, Regulons, and Global Regulators

In a bacterial **operon**, one or more genes are transcribed together as a single mRNA transcript initiated from a single promoter upstream of the genes. Genes in an operon typically are all regulated and expressed together. In a **regulon**, genes can be found in different locations on the bacterial chromosome, but all have promoters that are controlled by the same regulatory signaling protein, called a **regulator**. This regulator controls the expression of genes within multiple operons as well as multiple single genes. **Coordinate regulation** is the regulation of multiple genes in response to a particular condition or signal. When the regulon of a single regulator consists of a large number of genes that affect a broad range of physiological processes, the regulator is referred to as a **global regulator**. Coordinate regulation provides a framework for working out circuits of virulence gene control, as virulence genes are often contained within the same regulon and often share a common global regulator.

For example, the protein PrfA positively regulates the transcription of many of the genes involved in the virulence of the foodborne pathogen *Listeria monocytogenes* (see Figure 11-14 for the life cycle of *Listeria*). The PrfA regulon is comprised of three multigene operons and several single-gene operons (Figure 14-1). The first two multigene operons consist of the *plcA-prfA* genes and the *mpl-actA-plcB* genes, followed by three open reading frames of unknown function (*orfX-orfY-orfZ*). These two operons flank the single-gene *hly* operon, which is divergently transcribed from *plcA-prfA*. All of these genes are located in the *Listeria* pathogenicity island-1 (LPI-1). Northern blot analysis indicates that there are multiple promoters in this region that are regulated by PrfA, some of which are located in the intercistronic regions between genes that are also cotranscribed (e.g., between *plcA* and *prfA*). These internal promoters allow an additional level of transcriptional control by PrfA and possibly other regulators. The other three operons that are part of the PrfA regulon, one comprised of two genes (*inlA-inlB*) and the other two of one gene each (*inlC* and *hpt*), were identified at other locations on the chromosome and were shown to encode proteins involved in virulence.

Activators and Repressors

Transcription factors are proteins that bind to specific DNA sequences to control expression of genes in an operon or regulon. They can be separated into two types based on

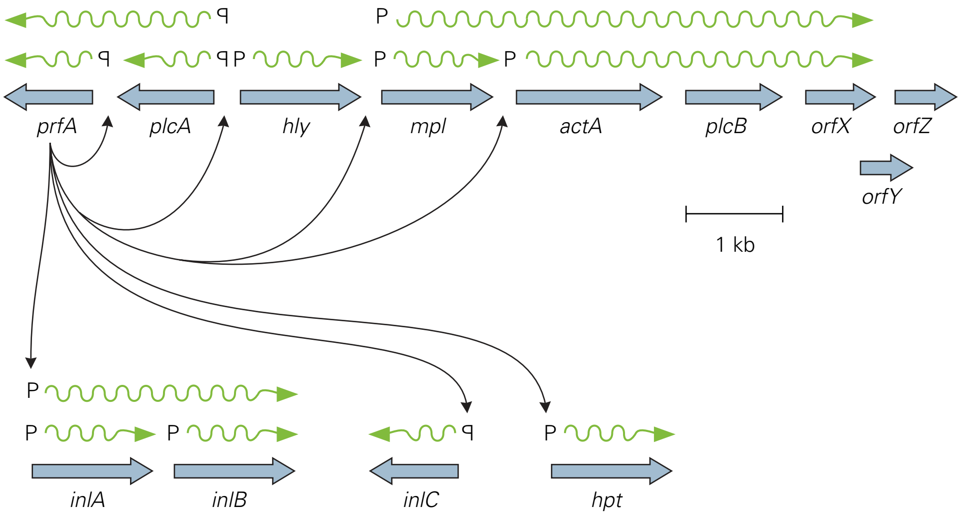


Figure 14-1. Organization and regulatory control of *Listeria monocytogenes* virulence genes that are members of the PrfA regulon. Shown is the core PrfA regulon involved in *L. monocytogenes* virulence. The gene *prfA* encodes the regulatory protein PrfA that activates genes in three multigene operons and several single-gene operons indicated by the arrows. The genes located in the *Listeria* pathogenicity island-1 (LPI-1) are: *hly* (encodes LLO, a pore-forming toxin critical for escape from the phagosome and double-membrane vacuoles); *plcA* (encodes PI-PLC, a phosphatidyl inositol-specific phospholipase that helps in escape from the phagosome and vacuoles); *plcB* (encodes PC-PLC, a phosphatidyl choline-specific phospholipase that helps in escape from the vacuoles); *actA* (encodes ActA, a protein that nucleates polymerization of host actin on the bacterial cell surface and causes intracellular motility); *mpl* (encodes Mpl, a zinc-dependent metalloprotease that processes PC-PLC precursor to its mature form); and *orfs X,Y,Z* (encode several genes of unknown function). Genes in the *inlAB* operon are *inlA* and *inlB* (encode InlA and InlB, adhesion proteins called internalins that contribute to invasion of host cells). The *inlC* operon contains a single gene: *inlC* (encodes InlC, an internalin-like protein that promotes protrusion formation at apical junctions and cell-to-cell spread). The *hpt* operon also contains a single gene: *hpt* (encodes Hpt, a hexose phosphate transporter required for optimal intracellular growth). Locations of promoters are indicated by "P"; wavy lines indicate mRNA transcripts.

whether they increase or decrease expression of operon or regulon genes. **Transcriptional activators** are transcription factors that stimulate the expression of the associated genes by recruiting RNA polymerase to promoters near their DNA-binding sites. Some transcriptional activators require a coactivator, ligand, or modification such as phosphorylation to bind to the DNA or to interact with the RNA polymerase. **Transcriptional repressors** serve the opposite function. They bind to specific DNA sequences in promoter regions and physically block RNA polymerase from binding to the promoter or progressing with transcription. The specific DNA sequences that repressors bind to are called **operators**. Similar to transcriptional activators, some transcriptional repressors require a corepressor, ligand, or modification to bind to the DNA. In some cases, repressors strongly bind to their operator sequences, but after binding to a specific ligand, they disassociate from the DNA, relieving the block in transcription.

The prototypical example of transcriptional regulation by the opposing actions of an activator and a repressor is the *lac* operon of *Escherichia coli*. This operon regulates the genes involved in the transport and metabolism of the

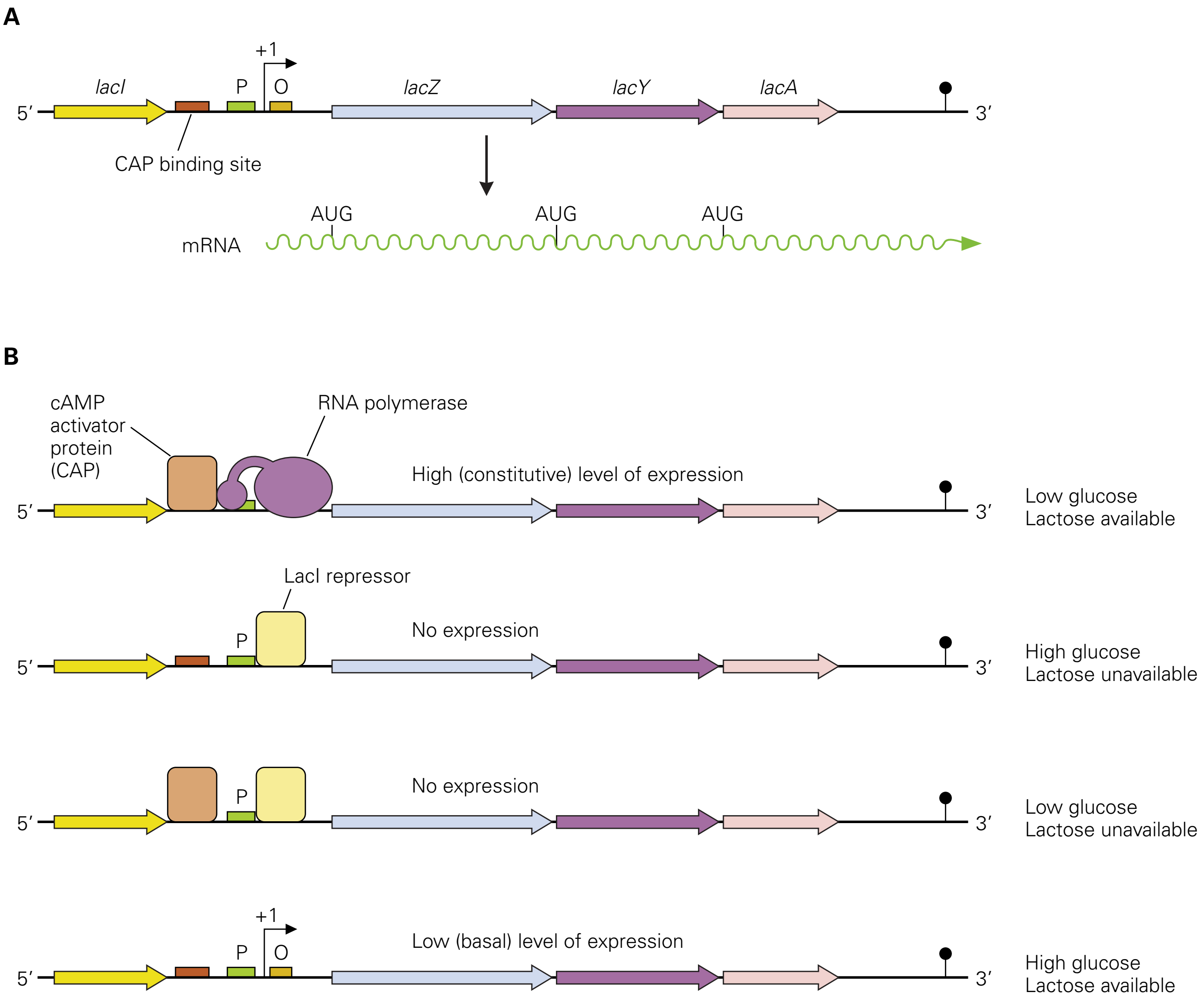

A
+1
5′
lacI
P
O
lacZ
lacY
lacA
3′
CAP binding site
AUG
AUG
AUG
mRNA
B
cAMP activator protein (CAP)
RNA polymerase
High (constitutive) level of expression
5′
3′
Low glucose
Lactose available
LacI repressor
P
No expression
5′
3′
High glucose
Lactose unavailable
P
No expression
5′
3′
Low glucose
Lactose unavailable
+1
P
O
Low (basal) level of expression
5′
3′
High glucose
Lactose available

Figure 14-2. Regulation of the *lac* operon of *Escherichia coli*. (A) The *lac* operon of *E. coli*. The *lacZ* gene encodes β-galactosidase, *lacY* encodes a permease, and *lacA* encodes a transacetylase. LacI is a repressor of the *lac* operon. The cAMP activator protein (CAP) binding site and the *lac* operator (O) are sites where regulatory proteins bind to control expression from the *lac* promoter (P). **(B)** In the absence of lactose, the LacI repressor protein binds to the operator, which overlaps the promoter, and prevents transcription of the *lacZYA* genes. For optimal transcription of the *lacZYA* genes, two conditions must be met: there must be sufficient lactose present so that an isomer of lactose (allolactose) can bind to the LacI repressor protein and release it from the promoter region, and there must be low levels of glucose so that cAMP is produced and binds to the cAMP activator protein, which in turn binds to DNA and recruits RNA polymerase to the *lac* promoter.

disaccharide, lactose (Figure 14-2). It consists of three cotranscribed genes under the control of a single promoter and an independently regulated, constitutively expressed transcriptional repressor LacI. The three cotranscribed genes are *lacZ* (encoding the enzyme β-galactosidase that hydrolyzes lactose into glucose and galactose monosaccharides), *lacY* (encoding a membrane-inserted, proton/sugar symporter permease that transports lactose into the cell), and *lacA* (encoding a transacetylase that can add an acetyl group to lactose). Glucose is the preferred carbon source of *E. coli*, so when glucose is present, the genes required for utilization of secondary carbon sources such as lactose are repressed. This phenomenon is called catabolite repression, and in Gram-negative bacteria, it is controlled through the production of cyclic adenosine monophosphate (cAMP). Because production of cAMP is inversely coupled to glucose uptake by the phosphoenolpyruvate:glucose phosphotransferase glucose-transport system, low glucose leads to high cellular cAMP. cAMP is the ligand for the transcriptional activator, cAMP activator protein (CAP). Once bound to cAMP, CAP binds to a site near the *lac* operon promoter where it can recruit RNA polymerase. However, absence of glucose alone is not sufficient to activate the *lac* operon, as when no lactose is present, the constitutively expressed LacI repressor protein binds to its cognate operator sites in the promoter region of the *lac* operon to prevent transcription. It is only when lactose is also present in the cell that its isomer, allolactose, binds to LacI, releasing it from the operator and enabling expression of the *lac* operon.

Many virulence genes are regulated by iron through the action of iron-binding transcriptional repressors that are homologous to the **ferric uptake repressor (Fur)** of *E. coli*. An example of iron regulation of virulence gene expression is the case of diphtheria toxin, produced by the Gram-positive bacterium *Corynebacterium diphtheriae* (see chapter 12 for details). The diphtheria toxin gene (*tox*) is carried by several different temperate (lysogenic) bacteriophages. The gene for the regulator of diphtheria toxin gene expression (*dtxR*) is located on the bacterial chromosome and is constitutively expressed (see Figure 12-13). **DtxR** is a Fur-like transcriptional repressor that senses the presence of ferrous iron (Fe^{2+}). When iron levels are high, the Fe^{2+}-DtxR complex binds to the operator region of the *tox* gene and represses gene transcription. When iron becomes the growth-rate limiting nutrient, which is the prevailing condition in the host (see chapter 3), Fe^{2+} dissociates from the repressor, which in turn dissociates from the DNA. This allows transcription of the *tox* gene, as well as other iron-uptake genes, such as a corynebacterial siderophore and other components of the high-affinity iron transport system.

Staphylococcus aureus produces a wide assortment of virulence factors that help it evade immune responses and acquire nutrients from the host during infections (see chapter 18). *S. aureus* senses its metabolic state through the action of the **catabolite control protein E (CcpE)**, which is a LysR-type transcriptional regulator that binds to citrate, the first intermediate of the tricarboxylic acid (TCA) cycle (Figure 14-3A). CcpE acts as a master regulator for the expression of various virulence factors, including proteins involved in the biosynthesis of the yellow pigment **staphyloxanthin** (involved in evading oxidative killing), of **staphyloferrin** (a citrate-containing siderophore used for iron acquisition), and of capsular polysaccharide (involved in serum resistance). CcpE also mediates the production of superantigens (used in immune evasion), fibronectin-binding proteins (used in adhesion), and a number of exoproteases (used for immune evasion and dissemination). For example, deletion of either *ccpE* or *citB* results in production of staphyloxanthin (Figure 14-3B). CcpE binds to the promoter of *citB* (Figure 14-3C), the gene that encodes aconitase (the second enzyme of the TCA cycle), and activates its transcription (Figure 14-3D). CitB is involved in conversion of citrate to isocitrate in the TCA cycle, which is important for regulating biosynthesis of capsule and staphyloxanthin. Since staphyloxanthin is synthesized from acetyl-coenzyme A (acetyl-CoA) and acetoacetyl-CoA via the mevalonate pathway, deletion of *citB* blocks the TCA cycle and diverts more acetyl-CoA flux to the mevalonate pathway, thereby increasing pigment biosynthesis. The excess citrate is also funneled into the biosynthesis of the citrate-containing siderophore staphyloferrin. Given that nutrient and iron acquisition is an important determinant for survival in the host, it is not surprising that changes in the TCA cycle, such as the flux of citrate, provide a survival advantage for *S. aureus* during infection.

Two-Component Regulatory Systems

The transcriptional control of many bacterial virulence and metabolic genes occurs through **two-component regulatory systems (TCSs)** (Figure 14-4). With the exception of *Mycoplasma* species, which have relatively small genomes, all other eubacteria contain varying numbers of TCSs depending on the species. For example, *Streptococcus pneumoniae* and *E. coli* strains typically contain 13 and 30 TCSs, respectively. The simplest version of a TCS is composed of a sensory **histidine kinase** and its cognate **response regulator**, which acts as a transcriptional activator or repressor, depending on the TCS. Most sensor histidine kinases are homodimeric transmembrane proteins consisting of an extracellular sensing domain and an intracellular histidine phosphotransfer domain. There are a wide variety of different sensing domains that can recognize various signals, including small molecules, peptides, ions, or stress conditions. Upon stimulation by these signals, the intracellular phosphotransfer domain undergoes autophosphorylation of specific histidine residues. The cognate response regulator to the sensor histidine kinase recognizes this phosphoryl-

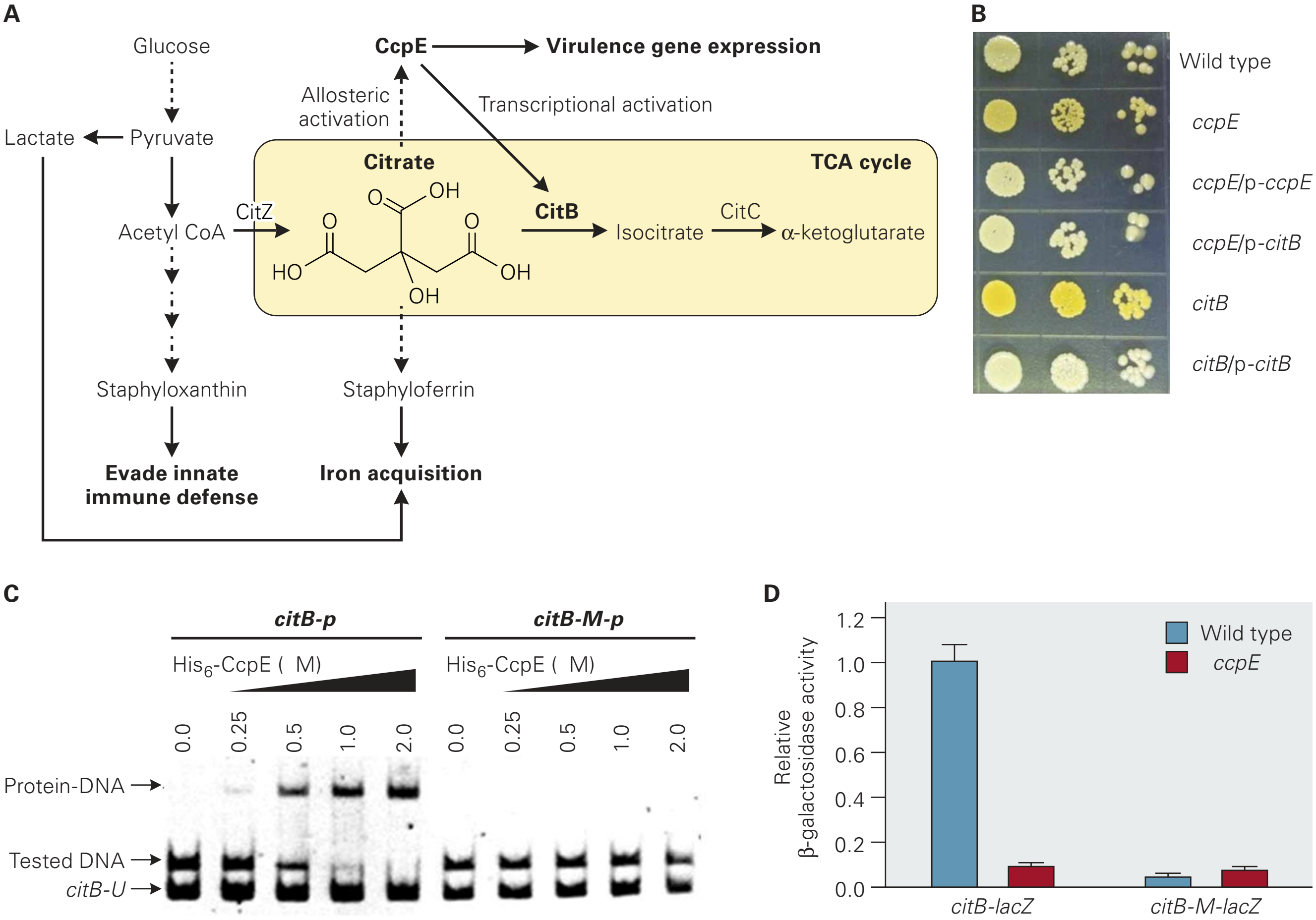

A
Glucose
Lactate
Pyruvate
Acetyl CoA
CitZ
CcpE
Virulence gene expression
Allosteric activation
Transcriptional activation
Citrate
TCA cycle
CitB
Isocitrate
CitC
α-ketoglutarate
Staphyloxanthin
Staphyloferrin
Evade innate immune defense
Iron acquisition
B
Wild type
ccpE
ccpE/p-ccpE
ccpE/p-citB
citB
citB/p-citB
C
citB-p
citB-M-p
His6-CcpE (M)
0.0
0.25
0.5
1.0
2.0
Protein-DNA
Tested DNA
citB-U
D
Relative β-galactosidase activity
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Wild type
ccpE
citB-lacZ
citB-M-lacZ

Figure 14-3. Metabolic sensing and virulence regulation in *Staphylococcus aureus*. (A) Proposed role of CcpE in metabolite sensing and virulence regulation. Through the use of citrate as a key inducer, *S. aureus* CcpE is involved in the regulation of TCA-cycle activity, the production of staphyloxanthin (used to evade oxidative killing), staphyloferrin (used for iron acquisition), and other virulence gene expression. CitZ, citrate synthase; CitB, aconitase; CitC, isocitrate dehydrogenase. CcpE negatively regulates the production of the pigment staphyloxanthin by activating the transcription of *citB*. CitB is the second enzyme of the TCA cycle and is responsible for the interconversion of citrate to isocitrate. Acetyl-CoA is also the precursor for staphyloxanthin production via the mevalonate pathway. Lactate facilitates the release of iron from the host iron-sequestering protein transferrin. **(B)** CcpE negatively modulates pigment production in the wild-type strain of *S. aureus* by activating the expression of the gene *citB*. The Δ*ccpE* and Δ*citB* strains are no longer repressed and so produce the pigment. Complementation of the genes through expression from a plasmid restores the negative control. **(C)** Assessment of mutation or deletion of the CcpE binding site. Electrophoretic mobility shift assays (EMSA) were performed using a wild-type fragment of the *citB* promoter DNA (*citB-p*) or the same fragment mutated (*citB-M-p*). *citB-U*, a control DNA fragment of the *citB* promoter DNA lacking a CcpE-binding region. **(D)** Expression levels of a transcriptional fusion reporter gene (*lacZ*) with a wild-type CcpE-binding site (*citB-lacZ*) or mutant (*citB-M-lacZ*). Panels B through D adapted from Ding Y, Liu X, Chen F, Di H, Xu B, Zhou L, Deng X, Wu M, Lan L. 2014. *Proc Natl Acad Sci USA* **111:**E4981–4990, with permission.

histidine residue and catalyzes the transfer of the phosphoryl group to a specific aspartate residue in the response regulator's receiver domain. Phosphorylation of the response regulator results in a conformational change in the protein's effector domain, which in turn modulates downstream cellular processes, usually through transcriptional activation or repression.

Many sensor histidine kinases have an additional phosphatase activity that can remove the phosphoryl group from their cognate response regulator to turn the system back off. Both phosphorylation and dephosphorylation can be controlled through the sensor domain. Additionally, some response regulators carry autophosphatase activity, which serves to limit how long they remain in an active state. Together, these phosphatase activities allow bacteria to fine-tune their responses to environmental stimuli while preventing aberrant crosstalk between different TCSs.

Although they are typically called "two-component" systems, this name is somewhat of a misnomer, as many of these systems actually involve multiple phosphorylation

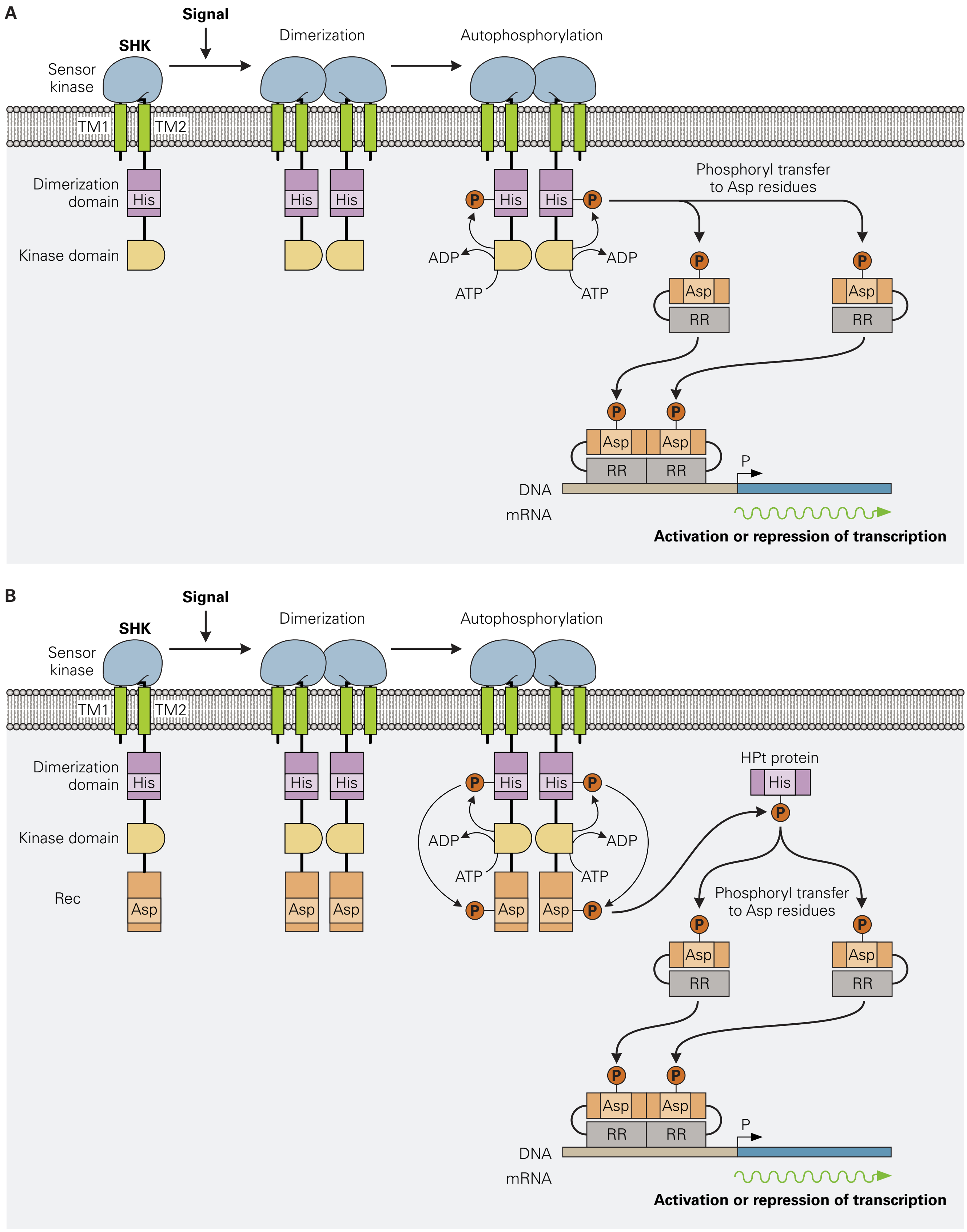

A
Signal
SHK
Sensor kinase
Dimerization
Autophosphorylation
TM1
TM2
Dimerization domain
His
Kinase domain
P
ADP
ATP
Phosphoryl transfer to Asp residues
Asp
RR
DNA
mRNA
Activation or repression of transcription
B
Signal
SHK
Sensor kinase
Dimerization
Autophosphorylation
TM1
TM2
Dimerization domain
His
Kinase domain
Rec
Asp
P
ADP
ATP
HPt protein
Phosphoryl transfer to Asp residues
RR
DNA
mRNA
Activation or repression of transcription

Figure 14-4. Two-component regulatory system for sensing and transducing extracellular signals. (A) Typical sensor histidine kinases (SHK) consist of an extracellular (Gram-positive) or periplasmic (Gram-negative) sensor domain, followed by a transmembrane domain, a dimerization domain, and an enzymatic kinase domain. Upon sensing a signal, the SHK dimerizes and undergoes autophosphorylation on a His residue of the dimerization domain. This phosphoryl group (P) can then be transferred to an Asp residue in the receiver (Rec) domain of the cognate response regulator (RR). **(B)** Alternatively, upon dimerization the phosphoryl group can be first transferred from the His residue to an Asp residue in the Rec domain of the protein. The phosphoryl group is then transferred to a His residue on the histidine phosphotransfer protein (HPt), which then serves as a transducer to transfer the phosphoryl group to the Rec domain of the RR. The phosphorylated RR then dimerizes, binds to the promoter region of certain genes, and activates (or sometimes represses) transcription of the genes, leading to a downstream cellular response to the signaling condition.

steps between the initial sensor histidine kinase and the terminal response regulator. Instead of being directly transferred to the response regulator, the phosphoryl group in these TCSs is passed through a series of histidine and aspartate residues present in multiple signaling domains within the sensor kinase or additional histidine-containing phosphotransfer (HPt; sometimes also called transducers) proteins (Figure 14-4). The sensor kinase, terminal response regulator, and intervening phosphotransfer domains or HPt proteins are collectively referred to as a **phosphorelay**. The BvgAS system, encoded by the operon *bvgAS*, is comprised of the sensor kinase BvgS and the response regulator BvgA. BvgAS is a prototypical phosphorelay (Figure 14-5A) that acts as a global regulator of virulence factor expression in *Bordetella pertussis* (Bvg stands for Bordetella virulence genes).

BvgAS regulation can be divided into three distinct phases based on the phenotypic manifestation of the bacteria during each phase, which in turn is determined by the levels of phosphorylated BvgA (Figure 14-5B). When growing stably at 37°C, the cells are in the Bvg$^+$ phase, and the sensor kinase BvgS is active, leading to the phosphorylation of the response regulator BvgA, which in turn positively regulates several processes, including surface adhesion, toxin production, and toxin secretion, while negatively regulating several others, most notably flagella assembly and motility. At lower temperatures (~25°C) or in the presence of $MgSO_4$ or nicotinic acid, the cells enter the Bvg$^-$ phase, where BvgS is inactive and BvgA remains unphosphorylated and unable to regulate transcription. It is important to note that although $MgSO_4$, nicotinic acid, and temperature modulate virulence gene expression in *Bordetella* that is

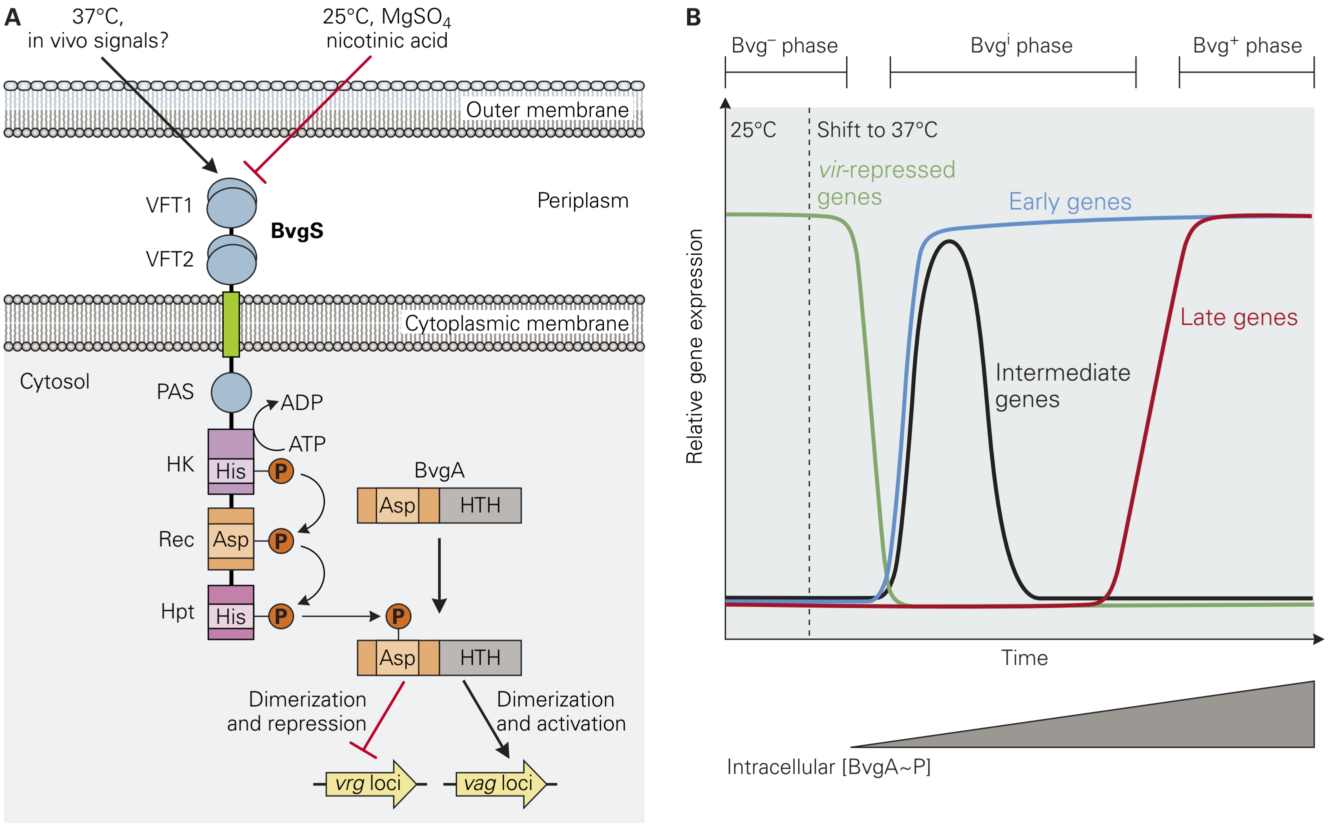

A
37°C,
in vivo signals?
25°C, MgSO4
nicotinic acid
Outer membrane
VFT1
VFT2
BvgS
Periplasm
Cytoplasmic membrane
Cytosol
PAS
ADP
ATP
HK
His
P
Rec
Asp
Hpt
His
BvgA
Asp
HTH
Dimerization
and repression
Dimerization
and activation
vrg loci
vag loci
B
Bvg⁻ phase
Bvgⁱ phase
Bvg⁺ phase
25°C
Shift to 37°C
vir-repressed
genes
Early genes
Late genes
Intermediate
genes
Relative gene expression
Time
Intracellular [BvgA~P]

Figure 14-5. The BvgAS phosphorelay two-component system serves as a master regulator of virulence genes in *Bordetella*. **(A)** BvgS is a multidomain histidine sensor kinase. At the N-terminus are two periplasmic Venus flytrap (VFT) domains and a cytosolic Pert-Arnt-Sim (PAS) domain. The VFT and PAS domains likely serve as the sensory domains. C-terminal to the PAS domain are three domains involved in the phosphorelay. They are a histidine kinase domain (HK), a receiver domain (Rec), and a histidine phosphotransfer domain (Hpt). In the presence of activating signals, a phosphoryl group (P) is transferred along the phosphorelay onto an aspartate residue on the Rec domain of response regulator BvgA to form phosphorylated BvgA (BvgA~P). BvgA~P activates *vag* (virulence associated genes) loci and represses *vrg* (virulence-repressed genes) loci. **(B)** BvgAS-regulated genes undergo temporal activation. BvgA~P activates its own expression, so intracellular BvgA~P concentration increases with time at 37°C. During the Bvg$^-$ phase, *vrg* genes (e.g., flagellar genes) are expressed. As the cells enter the Bvg^i phase, early genes (e.g., filamentous haemagglutinin, fimbriae, and *bvgAS* itself) are expressed along with intermediate genes (*bipA*). Finally, during the Bvg$^+$ phase, late genes, which are directly involved in virulence (e.g., toxins), are expressed.

grown in the laboratory, the actual signals sensed by the BvgAS system inside the host remain unknown. The third phase, Bvg^{i}, occurs at intermediate levels of modulating signals, such as in specific mutants affecting the kinase activity of BvgS or in the first few hours after switching from Bvg^{-} to Bvg^{+} conditions. The Bvg^{i} phase is thought to represent a transition state between Bvg^{-} and Bvg^{+} phases, as some of the genes turned on during the Bvg^{+} phase, specifically those involved in adhesion, are expressed while other genes, such as those involved in toxin production and secretion, remain off. Additionally, there is at least one gene expressed exclusively in the Bvg^{i} phase, *bipA,* which encodes an outer membrane protein of unknown function. The finding that Bvg^{i} phase genes are involved in adherence and not toxin production and secretion has led scientists to hypothesize that the Bvg^{i} phase is involved in survival during transmission between individuals or during the early stages of infection. However, the lack of a robust animal model for *B. pertussis* infection makes testing these hypotheses difficult *in vivo*. Finally, recall that expression of BvgS is subject to phase variation that temporarily locks *B. pertussis* into the BvgS^{-} state (Figure 7-4). This phase variation may preserve a subpopulation of *B. pertussis* that expresses different surface antigens during infection as a hedge against the immune system.

Sigma Factors

Sigma (σ) factors are a subunit of the RNA polymerase holoenzyme. Sigma factors direct RNA polymerase to bind to specific DNA promoter sequences upstream of transcription start sites. Besides recruiting RNA polymerase to promoter regions, sigma factors assist in DNA strand separation and transcription initiation. As a key component of transcription initiation, sigma factors are an effective avenue for simultaneously regulating the expression of a large number of bacterial genes. Sigma factors were originally named based on their molecular mass. For example, in Gram-negative bacteria, σ^{70} is a 70-kDa protein that is the primary sigma factor responsible for transcription of all the basic “housekeeping” genes necessary for growth and survival, while σ^{38} is a 38-kDa protein (also known as σ^{S} or RpoS) that is expressed during late stationary phase and directs transcription of genes that mediate various stress responses.

The number of sigma factors varies among bacterial species. For example, *S. pneumoniae* has only two, σ^{70} and ComX, which induces competence (see chapter 7), whereas *E. coli* has at least seven sigma factors and *Mycobacterium tuberculosis* appears to have at least 13 (Table 14-1). Each RNA polymerase complex contains only one of these sigma factors at any given time and will only transcribe genes with promoters recognized by the sigma factor.

Table 14-1. Sigma factors and their functions in *Escherichia coli* and *Mycobacterium tuberculosis*

E. COLI	REGULATORY FUNCTION	*MYCOBACTERIUM TUBERCULOSIS*	REGULATORY FUNCTION
σ^{70} (RpoD)	Housekeeping genes necessary for cell growth	SigA (σ^{A})	Housekeeping genes necessary for cell growth
σ^{54} (RpoN)	Response to nitrogen-limitation conditions	SigB (σ^{B})	Response to hypoxia, oxidative stress, cell envelope stress
σ^{38} (RpoS)	Response to starvation and/or stationary phase	SigC (σ^{C})	Virulence
σ^{32} (RpoH)	Response to heat shock	SigD (σ^{D})	Response to starvation and/or stationary phase; stringent response
σ^{28} (RpoF)	Flagellar gene expression	SigE (σ^{E})	Response to surface stress, heat shock, virulence
σ^{24} (RpoE)	Response to extreme cytoplasmic or extreme heat stress	SigF (σ^{F})	Biosynthesis of mycobacterial cell envelope
σ^{19} (FecI)	Regulates expression of the *fec* (ferric citrate) gene for iron transport.	SigG (σ^{G})	SOS response, survival during macrophage infection
		SigH (σ^{H})	Response to oxidative stress, heat shock
		SigI (σ^{I})	Unknown
		SigJ (σ^{J})	Response to oxidative stress
		SigK (σ^{K})	Unknown
		SigL (σ^{L})	Virulence, biosynthesis of phthiocerol dimycocerosate
		SigM (σ^{M})	Long-term *in vivo* adaptation

There are also **anti-sigma factors** that control the function of sigma factors. Anti-sigma factors regulate their cognate sigma factors posttranslationally by sequestering them away from the RNA polymerase. When a particular condition is encountered by the cell, the anti-sigma factor releases the sigma factor, allowing the sigma factor to bind RNA polymerase and direct transcription of appropriate response genes. In some cases, anti-sigma factors are themselves negatively-regulated posttranslationally by **anti-anti-sigma factors**.

In some pathogens, virulence genes are regulated by multiple sigma factors as part of a larger regulatory network. For example, the major virulence system of the plant pathogen *Pseudomonas syringae* is a type III secretion system (T3SS) that delivers toxic effectors into plant cells. This T3SS and its effectors (see chapter 13) are encoded by genes controlled by the alternative sigma factor HrpL. HrpL is in turn regulated by σ^{54} (also called σ^{N} or RpoN), which is responsible for regulating several cellular processes (Figure 14-6A). By regulating genes in such a cascade, the bacterium is able to apply multiple layers of conditional regulation to production of its virulence factors.

Another example of a regulatory network involving multiple sigma factors is the PrfA regulon that we introduced earlier in Figure 14-1. PrfA is a transcriptional activator for the σ^{70}-containing RNA polymerase and positively regulates several virulence genes in *L. monocytogenes*, including *hly* and *actA*. Expression of the *prfA* gene encoding PrfA is actually controlled by two different sigma factors (Figure 14-6B). Under stress conditions, σ^{B} is expressed and used by RNA polymerase to direct transcription of a set of genes that includes *prfA*. Once PrfA starts being produced, it will then positively regulate itself using σ^{70} RNA polymerase. This **positive feedback loop** allows PrfA expression to be maintained even after σ^{B} expression has ceased.

In *M. tuberculosis*, the regulatory cascade is even more complicated with multiple sigma factors regulating the transcription of other sigma factors, each with its own positive feedback loop, anti-sigma factor, and anti-anti-sigma factor (Figure 14-6C). Each sigma factor in turn regulates its own set of genes controlling a different set of cellular functions.

Transcriptional Terminators and Antiterminators

In bacteria, multiple genes are often encoded within a single mRNA transcript. As such, special mechanisms are required to prevent unnecessary RNA polymerase activity at the end of a given transcriptional unit. Bacteria have two distinct mechanisms for controlling transcription termination. The first are **Rho-dependent terminators**, which are found in many, but not all, bacterial pathogens. These terminators involve **Rho (ρ) factor**, which binds to specific sites (called *rut* sites for R̲ho u̲t̲ilization) in the nascent mRNA strand. Rho then uses its helicase activity to travel along the mRNA toward the 3′ end (toward the bound RNA polymerase). Normally, the RNA polymerase translocates along the DNA template faster than Rho translocates along the mRNA, so Rho is unable to affect the polymerase, but whenever the polymerase is slowed or stalled, Rho will catch up to the polymerase and disrupt the RNA polymerase-mRNA-DNA complex, thereby halting transcription.

Rho-independent terminators (also called intrinsic terminators) do not use Rho factor. Instead, secondary structures of the mRNA transcript form in the sequence itself. These RNA secondary structures consist of a hairpin loop with a GC-rich 7 to 10 base-pair stem followed by several uracil residues. When RNA polymerase encounters these hairpin structures in the nascent mRNA, the polymerase stalls, destabilizing the RNA polymerase-mRNA-DNA complex and halting transcription.

Terminators generally act as stop signals for the RNA polymerase to prevent transcription of nonsense RNA beyond the end of operons. However, in some cases, bypassing a terminator allows RNA polymerase to transcribe additional genes. This property is taken advantage of in some bacteria to regulate sets of genes by having specific **antitermination factors** that enable certain terminators to be bypassed. Many of these antiterminators prevent the formation of the terminator hairpin by stabilizing alternative secondary structures (Figure 14-7A). In some cases, a specific protein will mediate this process after binding to a metabolite ligand. In others, the mRNA itself will adopt the alternative secondary structure after directly binding the metabolite ligand. These metabolite-sensing RNAs are called **riboswitches** and typically control the biosynthesis of the ligand that they sense.

Since translation and transcription are coupled in bacteria, ribosomes trailing RNA polymerase that are in the process of translating the nascent mRNA can also act as antiterminators. When the trailing ribosome is actively progressing, it will not impose any restrictions on the mRNA secondary structure, allowing the terminator hairpin to form and transcription to terminate. By contrast, when the metabolic conditions are not favorable for active translation (e.g., depletion of a specific charged tRNA), the ribosome will stall at a specific location preventing the formation of the terminator hairpin (Figure 14-7B). This process is called **attenuation** and is frequently used to regulate amino acid biosynthesis in Gram-negative bacteria.

In most Gram-positive bacteria, regulation of amino acid biosynthesis and aminoacyl tRNA synthetase genes occurs independently from the ribosome. Instead, they have riboswitches called **T-boxes** that specifically bind to uncharged tRNAs and prevent terminator hairpin formation (Figure 14-7C). Bacteriophage also frequently use regulated transcription termination to regulate their lifecycle. These

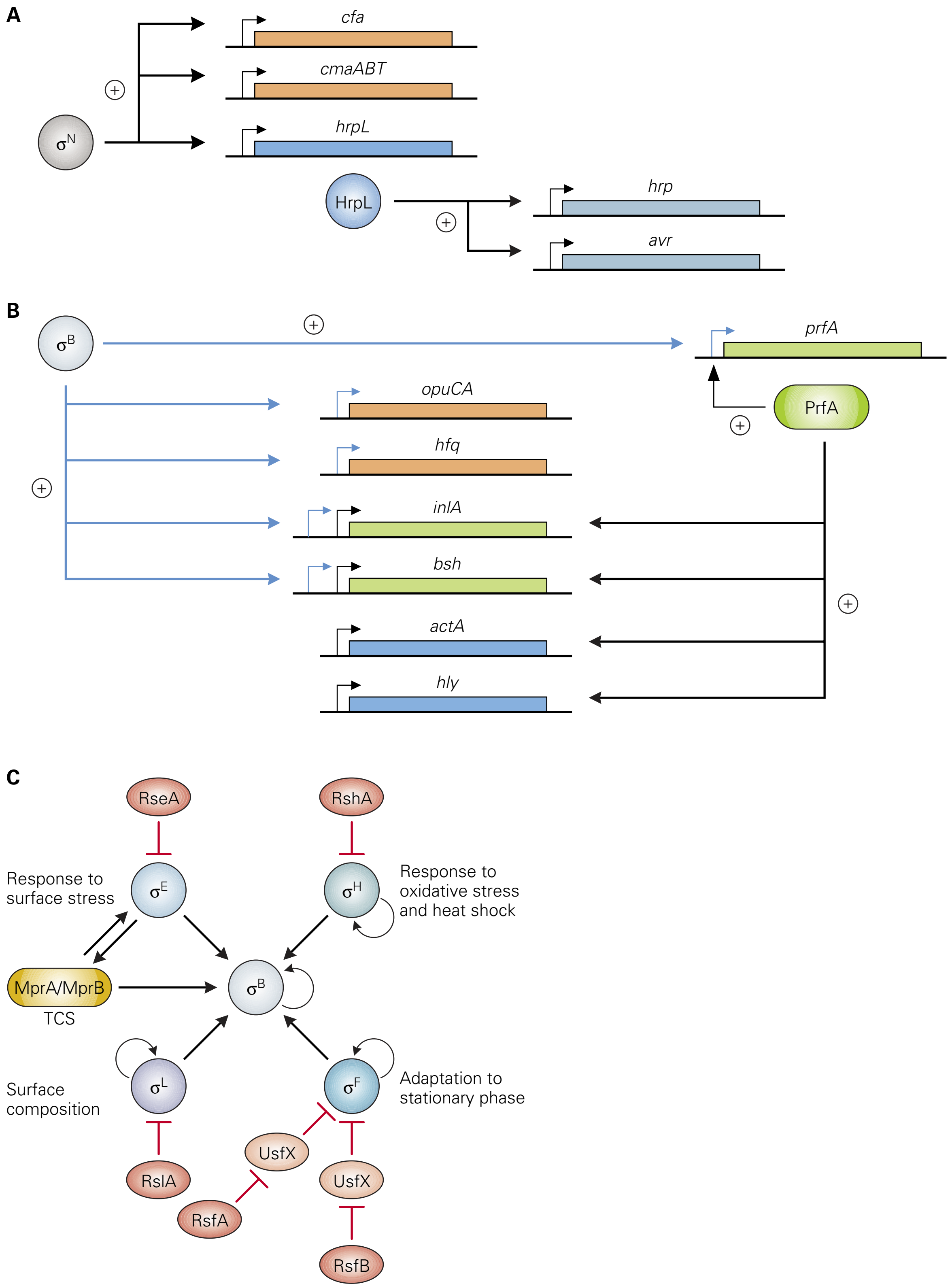

A
cfa
cmaABT
+
σN
hrpL
HrpL
+
hrp
avr
B
σB
+
prfA
PrfA
+
opuCA
hfq
+
inlA
bsh
+
actA
hly
C
RseA
RshA
Response to
surface stress
σE
σH
Response to
oxidative stress
and heat shock
MprA/MprB
TCS
σB
Surface
composition
σL
σF
Adaptation to
stationary phase
UsfX
UsfX
RslA
RsfA
RsfB

Figure 14-6. Examples of regulatory networks involving sigma factors and other transcriptional regulators. (A) The sigma factor cascade regulating expression of the type II secretion system (T2SS) in *Pseudomonas syringae*. Sigma-N mediates transcription of the *cfa* and *cma* operons (biosynthesis of polyketide components of the phytotoxin coronatine) and *hrpL*, which encodes a sigma factor (HrpL) responsible for transcriptional regulation of the *hrp* and *avr* genes involved in T2SS expression. **(B)** The PrfA-sigma-B network of *Listeria monocytogenes*. Sigma B (σ^B), which responds to general stress in Gram-positive bacteria, mediates increased expression of the PrfA transcriptional activator of virulence gene expression (see Figure 14-1). The gene *inlA* encodes internalin A, *hly* encodes LLO involved in escape from phagolysosome, *actA* encodes ActA involved in actin tail formation, *bsh* encodes a bile salt hydrolase involved in *Listeria* resistance to bile, *hfq* encodes an RNA-binding protein chaperone important for modulating stability or translation of mRNAs and interacts with numerous regulatory sRNAs, and *opuCA* encodes a solute transporter. **(C)** The complex interaction of several sigma factors (see Table 14-1) involved in virulence of *Mycobacterium tuberculosis*.

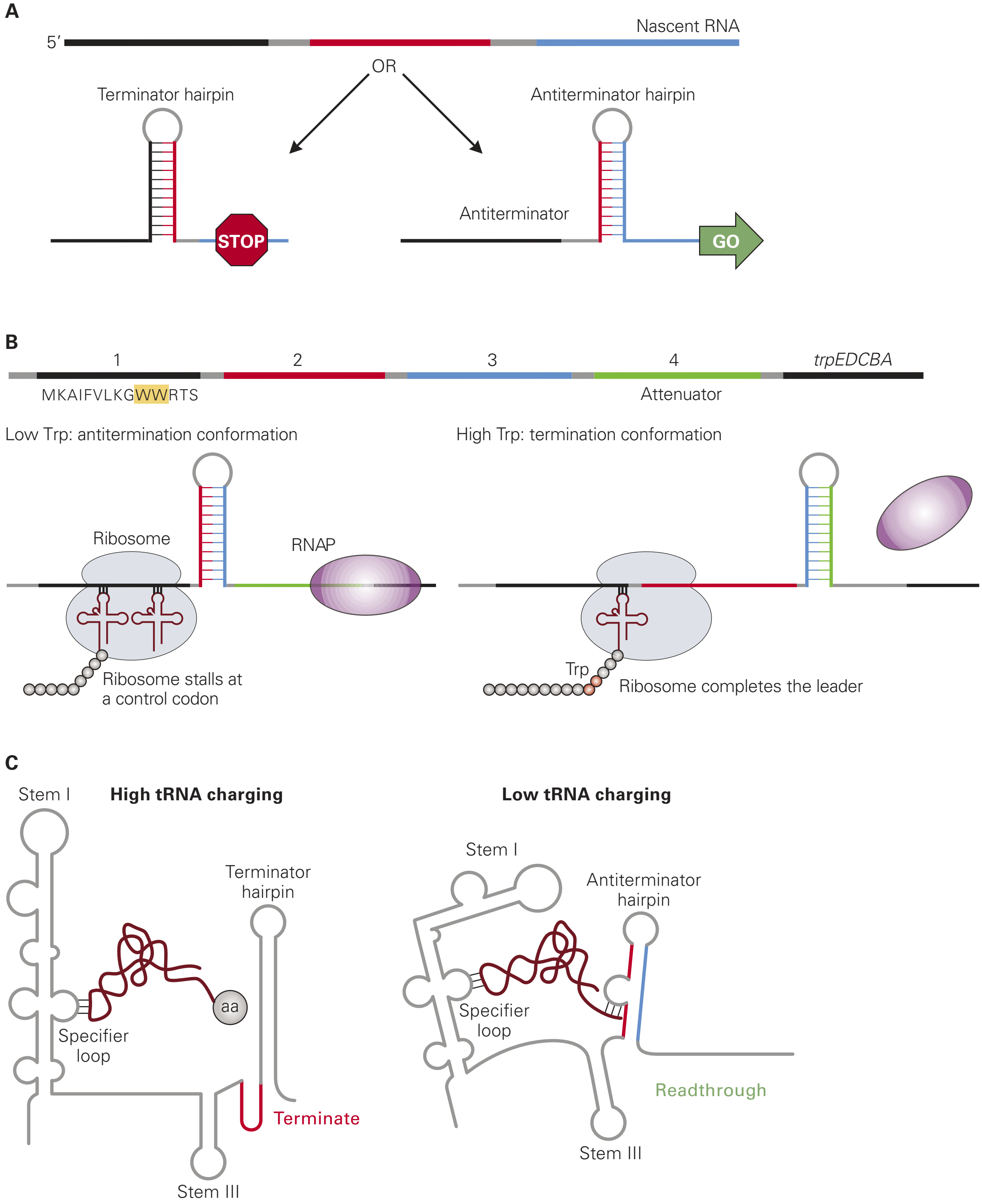

A
5′
Nascent RNA
OR
Terminator hairpin
Antiterminator hairpin
Antiterminator
STOP
GO
B
1
2
3
4
trpEDCBA
MKAIFVLKGWWRTS
Attenuator
Low Trp: antitermination conformation
High Trp: termination conformation
Ribosome
RNAP
Ribosome stalls at
a control codon
Trp
Ribosome completes the leader
C
Stem I
High tRNA charging
Low tRNA charging
Stem I
Terminator
hairpin
Antiterminator
hairpin
aa
Specifier
loop
Specifier
loop
Terminate
Readthrough
Stem III
Stem III

Figure 14-7. Antiterminators mechanism. (A) Basic schematic of an antitermination mediated by a protein. In this case, a terminator hairpin will form unless an RNA-binding anti-terminator protein binds and blocks formation of the hairpin. **(B)** An antiterminator can serve as part of attenuation of an operon. Shown is the attenuation of the *trp* operon in *E. coli*, where the region upstream of the structural *trp* genes encodes a 14-residue leader peptide (region 1). Region 3 can base pair with region 2 to form an anti-terminator hairpin or with region 4 to form a terminator hairpin. When Trp levels are low, the ribosome stalls at the sites in the leader peptide region where there are two Trp codons (WW), thereby causing the antiterminator hairpin to form, which results in expression of Trp biosynthetic genes (*trpEDCBA*) and more Trp in the cell. When Trp levels are high, the ribosome passes through the WW codons, progresses into the region 2 and completes the leader peptide. This blocks the antiterminator hairpin from forming and instead allows the terminator hairpin to form, and the RNA polymerase (RNAP) to dissociate. Based on information from Santangelo TJ, Artsimovitch I. 2011. *Nat Rev Microbiol* **9**:319–329. **(C)** T-box riboswitch. When the tRNA is charged with an amino acid, it will only bind to the specifier loop. When the tRNA is uncharged, it interacts with both the specifier loop and the antiterminator region (red), thereby stabilizing the antiterminator hairpin and allowing the terminator to be bypassed. Adapted from Green NJ, Grundy FJ, Henkin TM. 2009. *FEBS Lett* **584**:318–324, with permission.

transcription terminators, unlike those discussed above, often act on the RNA polymerase itself enhancing its processivity, allowing it to bypass multiple transcription terminators. Although transcription termination mechanisms are important for controlling the underlying metabolism of most bacteria, including pathogens, these mechanisms are not typically employed in the regulation of virulence factors themselves.

Regulation of Translation Initiation

In the previous section we discussed how RNA secondary structures play a role in regulating the formation of the mRNA transcript. As it turns out, RNA secondary structures also play a role in regulating the translation of these transcripts into proteins. In order for translation initiation to occur, ribosomes bind the mRNA in the 5′ region immediately upstream of the start codon of a gene at a specific ribosome-binding site (RBS; also called Shine-Dalgarno [SD] sequence in bacteria). However, RNA secondary structures can sequester these sites, preventing ribosomes from accessing them. Similar to the hairpin terminators discussed in the previous section, these secondary structures can be altered to expose the RBS through small molecule ligand riboswitches (Figure 14-8A) or through binding of specific regulatory proteins (Figure 14-8B). These altered secondary structures can also sequester or expose RNase cleavage sites, respectively increasing or decreasing the stability of the transcript.

In some cases, RNA secondary structures can serve as sensors of temperature changes. Instead of binding small molecule ligands, **thermostable riboswitches** form stable secondary structures at low temperatures that melt at higher temperatures, such as the *E. coli* RNA thermosensor within the *rpoH* gene (heat shock sigma factor σ^{32}) (Figure 14-8C). LcrF is the primary transcriptional activator of several virulence genes in *Yersinia pestis*, the causative agent of plague. At low temperatures, the RBS near the 5′-end of the *lcrF* mRNA is sequestered in a hairpin-loop structure that forms in the intergenic region. The stability of the hairpin structure decreases with increasing temperature, resulting in more expression of the LcrF protein at 37°C than at lower temperatures (Figure 14-8D). This kind of temperature sensing likely operates in many zoonotic pathogens, like *Y. pestis*, whose infection life cycle includes both cold- and warm-blooded hosts (fleas and mammal in this case). Thermostable riboswitches also operate in other pathogens that grow in the environment at lower temperatures before infecting human hosts. For example, translation of the PrfA virulence regulatory protein of *Listeria* is increased at higher temperatures because of the RNA thermosensor that forms a hairpin secondary structure in the untranslated 5′-end of the *prfA* mRNA transcript (Figure 14-8E). Mutations that destabilize the structure increase PrfA protein levels at lower temperatures, thereby enabling expression of virulence factors and invasion of host cells.

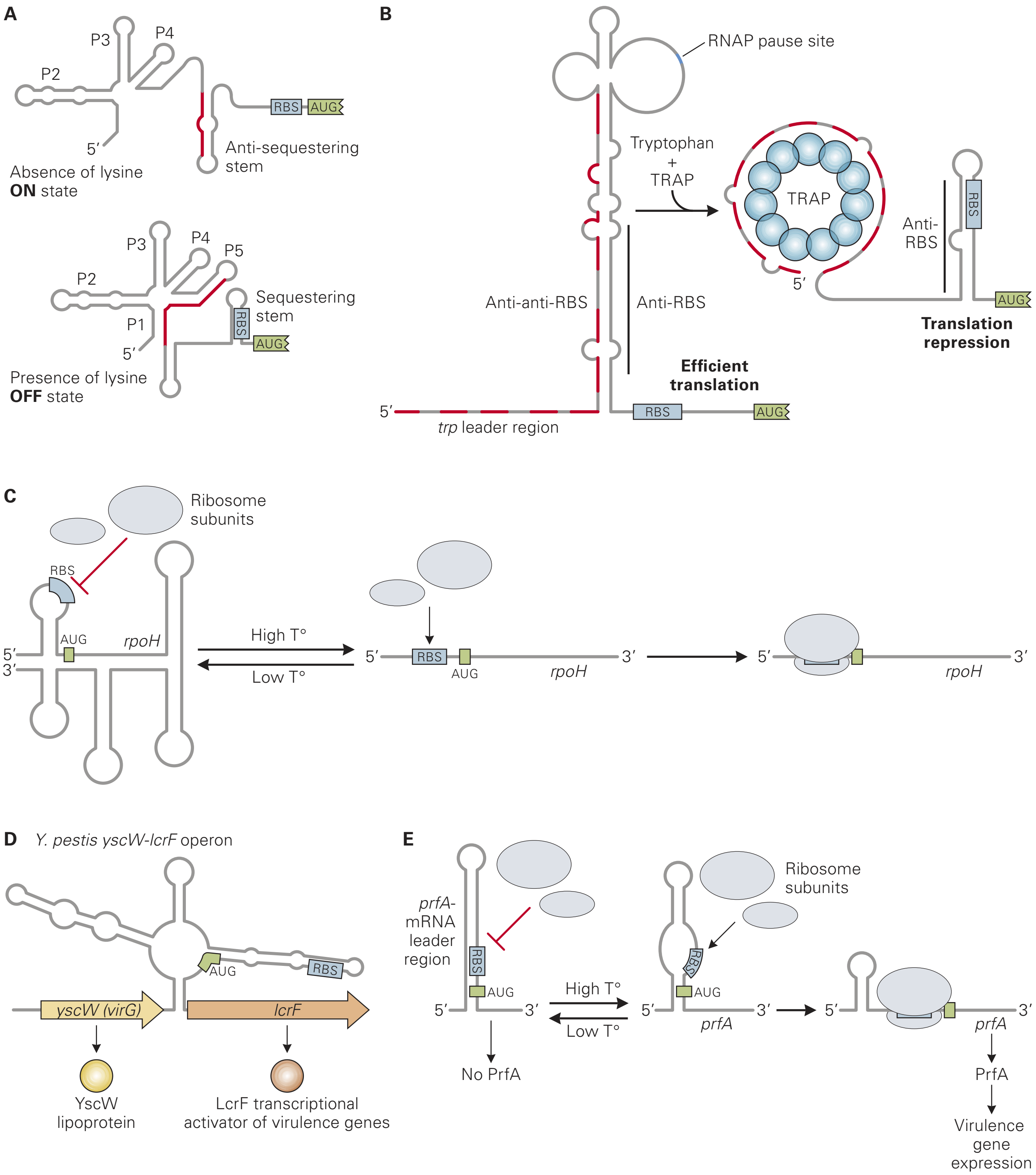
Regulatory Small RNAs

In addition to mRNAs, which serve as the coded template for protein translation, bacteria also generate small RNA transcripts. These **small RNAs (sRNAs)** typically range from 50 to 500 nucleotides in length and serve to regulate a number of different cellular processes. sRNAs can generally be grouped into two different categories, those that bind directly to mRNA transcripts to affect translation, transcription termination, or mRNA stability (**base-pairing sRNAs**) and those that bind directly to DNA or RNA-binding proteins (**protein activity-modifying sRNAs**) to modulate their enzyme activity and thereby regulate processes. Moreover, it was recently discovered that some sRNAs function both by base pairing to mRNAs and by encoding small regulatory proteins.

Base-pairing sRNAs. In many cases, base-pairing sRNAs bind to mRNA transcripts to inhibit translation. In Gram-negative bacteria, the protein **Hfq** serves as a chaperone to protect these sRNAs from degradation and to mediate their binding to the mRNA transcripts. However, in some Gram-positive bacteria there are sRNAs that do not require Hfq to function, although there may be yet-to-be-found additional RNA chaperones. Base-pairing sRNAs can block translation by preventing the ribosome from accessing the mRNA. In some cases, binding to the mRNA transcript can also stimulate degradation of the transcript, further enhancing the negative regulatory action. On the other hand, base-pairing sRNAs can also serve to enhance translation by preventing the formation of inhibitory secondary structures in the mRNA transcript like the riboswitches discussed in the previous section.

An example of an sRNA exhibiting both negative and positive regulatory effects is DsrA from *E. coli.* DsrA is an Hfq-dependent, 85 nucleotide long noncoding sRNA. Two regions of DsrA are separately complementary to two different mRNAs (Figure 14-9A). DsrA negatively regulates translation of the bacterial nucleoid protein H-NS (discussed later in this chapter) by base pairing immediately downstream of the start codon to block translation initiation and promote RNase-mediated degradation of the transcript. In addition, DsrA positively regulates translation of the stationary-phase sigma factor RpoS (discussed above) by binding to and opening up a hairpin in the mRNA of RpoS that normally inhibits its translation.

The Hfq-dependent base-pairing sRNAs discussed so far are typically encoded separately from the genes that they regulate. However, there is a class of sRNAs that is generated from transcription of the antisense strand of certain genes. These **antisense sRNAs**, also called *cis*-encoded sRNAs, typically regulate the gene that they arise from. Among


A
P2
P3
P4
5′
RBS
AUG
Anti-sequestering stem
Absence of lysine
ON state
P2
P3
P4
P5
P1
5′
Sequestering stem
RBS
AUG
Presence of lysine
OFF state
B
RNAP pause site
Tryptophan
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TRAP
TRAP
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RBS
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Translation repression
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Ribosome subunits
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3′
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D
Y. pestis yscW-lcrF operon
AUG
RBS
yscW (virG)
lcrF
YscW lipoprotein
LcrF transcriptional activator of virulence genes
E
prfA-mRNA leader region
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High T°
Low T°
Ribosome subunits
RBS
AUG
5′
3′
prfA
5′
3′
prfA
PrfA
Virulence gene expression

Figure 14-8. Model mechanism for how riboswitches can control translation initiation. (A) The *lysC* riboswitch of *E. coli* modulates the mRNA level upon lysine binding. In the absence of lysine, an antisequestration hairpin forms that exposes the ribosome binding site (RBS) to allow translation initiation. When lysine binds to the RNA riboswitch, a new secondary structure forms in which the RBS is sequestered in a hairpin, thereby preventing translation. **(B)** Translational repression of tryptophan metabolism gene *trpE* in *Bacillus subtilis* is mediated by *trp* RNA binding attenuation protein (TRAP). When tryptophan is limiting, the *trpE* mRNA adopts a structure such that the RBS is available for ribosome binding. When tryptophan is in excess, TRAP binds tryptophan and becomes activated and binds to sites near the RBS, which promotes the formation of an RBS-sequestering hairpin that prevents translation. **(C)** In *E. coli*, expression of the heat shock sigma factor σ^{32} is controlled by an RNA thermostable riboswitch within the *rpoH* gene, which forms a secondary structure within the translation-initiation region at low temperatures (<30°C). This inhibitory structure is disrupted at high temperatures (>42°C), resulting in translation of *rpoH*. **(D)** The 124-nucleotide intergenic region between *yscW,* which encodes a lipoprotein, and *lcrF,* which encodes a global regulator of virulence in *Yersinia pestis,* forms a secondary structure that serves as an RNA thermosensor. At body temperatures of 37°C, this structure opens up to permit ribosome binding to the RBS site and translation of *lcrF*. **(E)** Expression of the transcriptional activator of *Listeria* virulence genes (*prfA*) is high at 37°C and low at 30°C. This thermosensor-regulation requires a hairpin structure at the untranslated 5′-end of the *prfA* mRNA transcript. Panel A adapted from Caron MP, Bastet L, Lussier A, Simoneau-Roy M, Massé E, Lafontaine DA. 2012. *Proc Natl Acad Sci USA* **109:**E3444–3453, with permission. Panel B data from Babitzke P, Baker CS, Romeo T. 2009. *Annu Rev Microbiol* **63**:27–44. Panel C data from Kortmann J, Narberhaus F. 2012. *Nat Rev Microbiol* **10**:255–265. Panels D and E data from Krajewski SS, Narberhaus F. 2014. *Biochim Biophys Acta* **1839**:978–988.

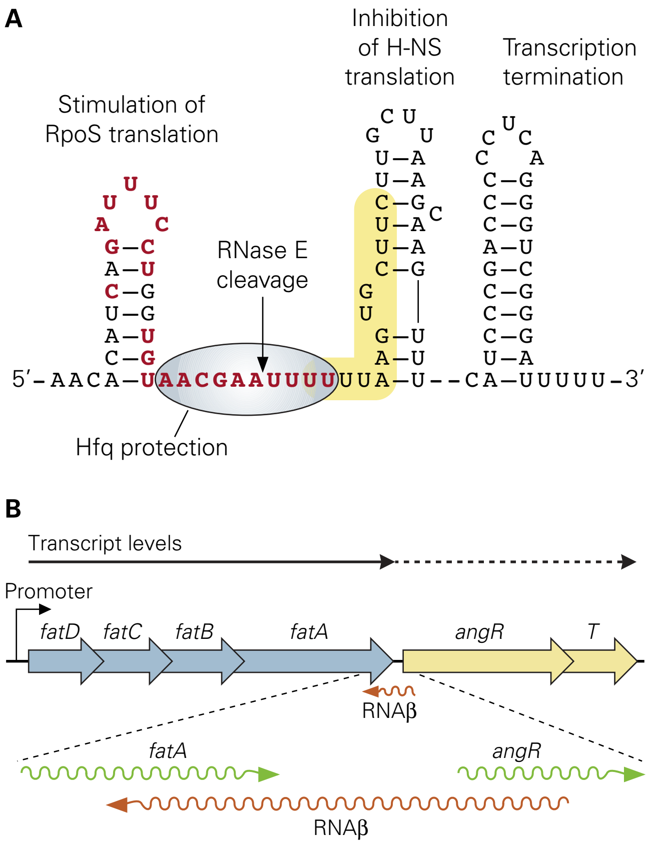

A
Stimulation of RpoS translation
Inhibition of H-NS translation
Transcription termination
RNase E cleavage
Hfq protection
5′-AACA-UAACGAAUUUUUUA-U--CA-UUUUU-3′
B
Transcript levels
Promoter
fatD
fatC
fatB
fatA
angR
T
RNAβ
fatA
angR
RNAβ

Figure 14-9. Translation regulation by complementary regulatory RNAs. Many regulatory RNAs regulate translation or mRNA stability by base pairing with a complimentary sequence in the target mRNAs to form duplexes. Small RNAs (sRNAs) are synthesized starting at sites distant from the target and form imperfect base pairing with their targets, which is facilitated by RNA chaperone proteins, such as Hfq. **(A)** Shown is the sequence and secondary structure of the DsrA sRNA that positively regulates the stationary-phase sigma factor RpoS and negatively regulates the histone-like protein H-NS. Hfq binding to the site indicated (grey oval) protects DsrA from RNaseE cleavage. Hfq binding also facilitates DsrA binding to complementary target sequences (red letters for *rpoS* and yellow shading for *hns*) near the ribosome binding site of the target mRNAs. Data from Lease RA, Belfort M. 2000. *Proc Natl Acad Sci USA* **97**:9919–9924. **(B)** Antisense RNAs are synthesized from the opposite strand as the target RNA and involve long base-paired regions. For example, RNAβ is an antisense sRNA that regulates differential expression of genes in the *fat-ang* operon in *Vibrio anguillarum*. Data from Stork M, Di Lorenzo M, Welch TJ, Crosa JH. 2007. *J Bacteriol* **189**:3479–3488.

the first antisense sRNAs to be discovered were those that regulate certain mobile genetic elements through the repression of transposase synthesis (see chapter 7). Subsequently, several other genes have been shown to have complementary antisense sRNAs. For example, the fish pathogen *Vibrio anguillarum* carries a six-gene iron-uptake system on a 65-kb virulence plasmid (Figure 14-9B). The first four genes, *fatDCBA*, encode proteins involved in siderophore transport, while the last two genes, *angRT*, encode proteins involved in siderophore biosynthesis. Despite being cotranscribed as part of the same operon, mRNA levels of the *fat* genes are significantly higher than those of the *ang* genes. This differential expression is mediated by an antisense sRNA called RNAβ, which is transcribed from the antisense strand complementary to the 5′ end of *angR* and the 3′ end of *fatA*. As the *fatDCBA-angRT* mRNA transcript is made, secondary structures that arise from RNAβ binding to the mRNA result in premature transcription termination immediately after the *fatA* transcript is made.

Protein activity-modifying sRNAs. As the name implies, protein activity-modifying sRNAs act by binding to and modifying the activity of target proteins. Typically, this activity is simply the protein's ability to bind to a specific RNA or DNA target. In these cases, the sRNA competes with RNA or DNA targets for binding to the regulated enzymes. The best-studied of such a regulatory system is the CsrA family of translational repressors. In *E. coli*, CsrA is a critical regulator of carbon metabolism and storage, but its homologs in other bacteria regulate a variety of cellular processes, including virulence factor production, quorum sensing (discussed in more detail later in this chapter), motility, and biofilm formation. Typically, CsrA is a negative regulator that acts as a translational repressor by binding to the 5′ upstream region of regulated mRNA transcripts, thereby preventing ribosomes from accessing RBSs. The activity of CsrA is regulated by two sRNAs, CsrB and CsrC, which are 268 and 245 nucleotides in length, respectively. Both of these sRNAs contain multiple copies of a GGA sequence motif at the loop end of stem-loop structures, to which CsrA binds (Figure 14-10A). CsrB and CsrC bind to multiple copies of the CsrA protein through these motifs, effectively sequestering CsrA away from the mRNAs it normally targets.

Another well-studied member of the CsrA protein family is RsmA from *Pseudomonas aeruginosa*. *P. aeruginosa* is an opportunistic pathogen frequently associated with nosocomial infections in patients with compromised defenses, such as those with cystic fibrosis, neutropenia, or severe burns (see chapter 19). *P. aeruginosa* infections can be divided into two phases: acute infection, where the bacteria rapidly spread systemically after entering the host, causing mortality within days or weeks, and chronic infection, where the bacterium persists in the host for years or decades. During each type of infection different bacterial virulence processes are employed. For example, during acute infection, genes associated with motility and the T3SS (see chapter 13) are upregulated, while during chronic infection, there is an upregulation of genes associated with secondary metabolism, biofilm formation, and the type VI secretion system (see chapter 13).

The RsmA protein is one of the major players that controls switching between these two states by negatively reg-

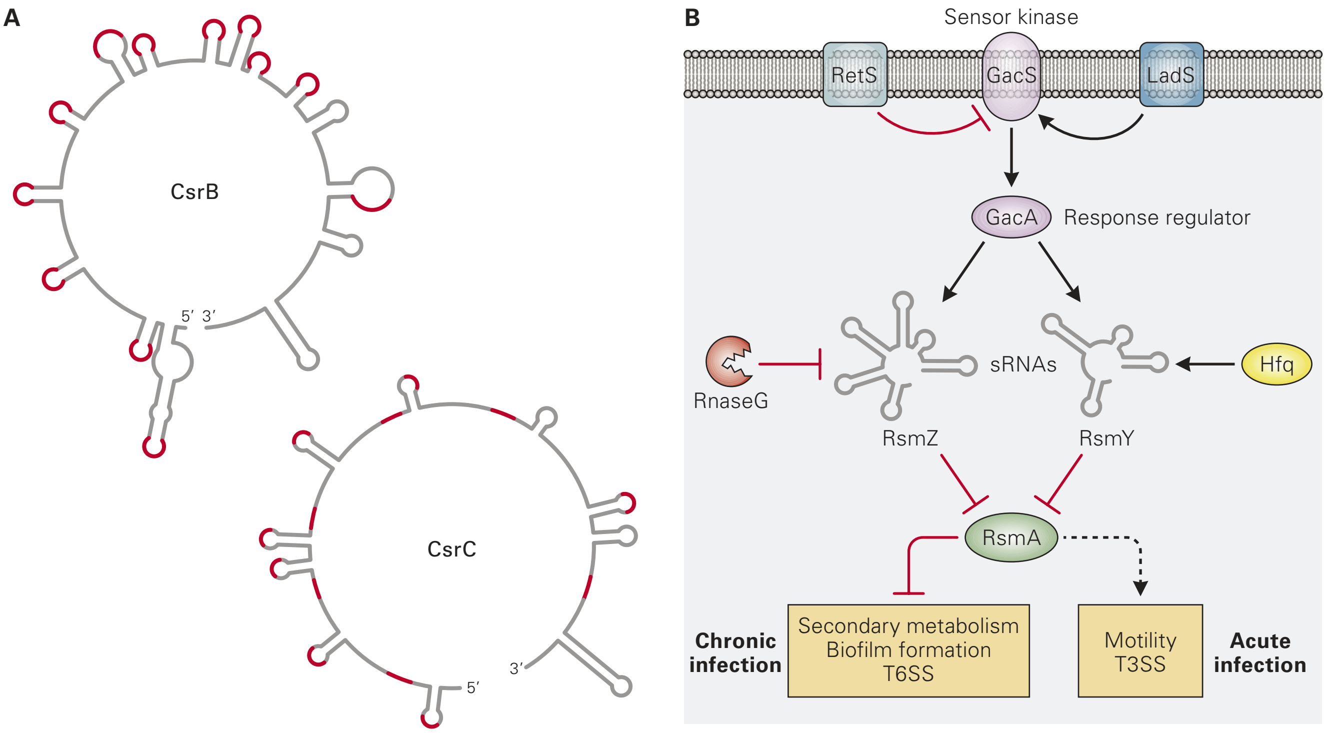


Figure 14-10. Translation regulation by protein activity-modifying sRNAs. Some sRNAs stimulate translation of target genes indirectly by sequestering away translation repressors. In so doing, they also help stabilize transcripts that would be subject to degradation when not covered by active ribosomes. **(A)** Regulation of CsrA in *E. coli* through sequestration by CsrB and CsrC. CsrB and CsrC carry numerous CsrA binding sites (indicated in red). The high frequency and density of these sites effectively sequester CsrA away from its normal mRNA targets. **(B)** The RsmA pathway of *Pseudomonas aeruginosa* controls the switch from acute to chronic infection. Activation of the global regulatory two-component system, GacSA via phosphorelay between the sensor kinase GacS and its cognate response regulator GacA results in transcription of the sRNAs, RsmY and RsmZ. RsmA is in the same family as CsrA. RsmY and RsmZ bind to RsmA and act to sequester RsmA away from its target gene mRNAs. RsmY is stabilized by binding to the RNA chaperone Hfq or to both Hfq and RsmA, which protects the complexes from degradation by RNase E. The amount of RsmZ is regulated by the levels of RNase G, which specifically cleaves RsmZ but not RsmY. Panel B data from Sonnleitner E, Romeo A, Bläsi U. 2012. *RNA Biol* **9**:364–371.

ulating chronic infection genes. Like CsrA, RsmA binds to GGA motifs near the RBS of target mRNAs, and the amount of free RsmA is negatively regulated by two sRNAs, RsmY and RsmZ, that bind to RsmA and sequester it away from its mRNA targets. Expression of the RsmY and RsmZ sRNAs is controlled by the two-component system GacSA and two associated sensor histidine kinases, RetS and LadS (Figure 14-10B). The two sRNAs are further regulated posttranscriptionally, with RsmY being stabilized through Hfq binding and RsmZ being degraded by RNase G. Altogether, the Gac/Rsm pathway enables large sets of genes to be coordinately activated in response to multiple environmental signals sensed by these two-component systems.

Bacterial Chromatin

The general perception of many scientists has long been that while eukaryotic chromosomes were organized into complex, ordered structures, bacteria were mere "bags of DNA and protein." However, as our understanding of bacterial cell biology has expanded in recent years, this perception has been completely overturned. Like eukaryotic chromosomes, bacterial chromosomes are organized into a higher-order structure called bacterial chromatin or the **nucleoid**. This structure facilitates the compaction and packaging of DNA into a cell that is only a thousandth of its linearized length, while still allowing essential processes such as replication, sister chromatid segregation, and gene expression to occur. Bacterial chromatin structure is shaped by various **nucleoid-associated proteins (NAPs)**, which bind chromosomal DNA either to locally twist and bend it or to bridge it with other sections of the chromosome.

As modulators of chromatin structure, NAPs also play an important role in gene regulation. One such NAP is the histone-like nucleoid structuring (**H-NS**) protein of *E. coli*. H-NS binds to AT-rich regions of DNA and polymerizes along the DNA molecule into filaments that interfere with gene transcription, either by preventing RNA polymerase from accessing genes or by blocking elongation of the transcript. Because these filaments can cover a large stretch of

DNA, this form of gene silencing is employed by bacteria to regulate entire operons or genomic islands.

For example, enteropathogenic *E. coli* (EPEC) (see chapter 19) carries a pathogenicity island called the locus of enterocyte effacement (LEE). This island consists of five major operons, *LEE1* to *LEE5*, and encodes a T3SS necessary for host colonization. At ambient environmental temperatures (27°C), H-NS silences the entire island. However, at higher temperatures indicative of having entered the host (37°C), H-NS silencing of the *LEE1* operon is relieved. This temperature sensing is probably due to H-NS filaments becoming unstable and disassociating at the higher temperature. It is not fully understood why the elevated temperature affects the silencing of only the *LEE1* operon, but in order to remove the silencing of the rest of the pathogenicity island, expression of the first gene in the *LEE1* operon, *ler*, is required. Ler is a paralog of H-NS that acts as an H-NS antagonist and specifically relieves the H-NS silencing of the remaining *LEE* operons.

Responding to Environmental Signals

In the previous section, several mechanisms behind gene regulation were discussed. In this section, we consider how different regulatory mechanisms can combine together to generate different bacterial phenotypes and behaviors, ultimately allowing bacteria to respond to their external environment.

Phase Variation and Bistable Switches

Phase variation refers to a particular type of phenotypic switching that occurs in bacterial populations. The two defining characteristics of phase variation are that the phenotypic changes must be *heritable*, allowing a cell to pass its altered state (or phase) to its progeny, and *reversible*, allowing for later generations to revert back to the original state. In the context of virulence, phase variation usually refers to the ability of a bacterial population to rapidly switch between "on" and "off" states of a gene or set of genes that allow them to survive inside the host. Typically, these genes encode surface structures, such as capsules, flagella, pili, cell wall components, lipoproteins, and lipopolysaccharides, or affect surface-related cellular processes, including adhesion, aggregation, and motility.

Phase variation that leads to variability in the bacterial components ultimately presented to the host immune system is known as **antigenic variation**. A high degree of antigenic variation can prevent the host from mounting an effective immune response (see the discussion of phase variable pili in *Neisseria gonorrhoeae* in chapter 7). Although antigenic variation is very beneficial to bacterial survival, unfortunately for the host, it presents a significant challenge for the development of effective vaccines (see chapter 17).

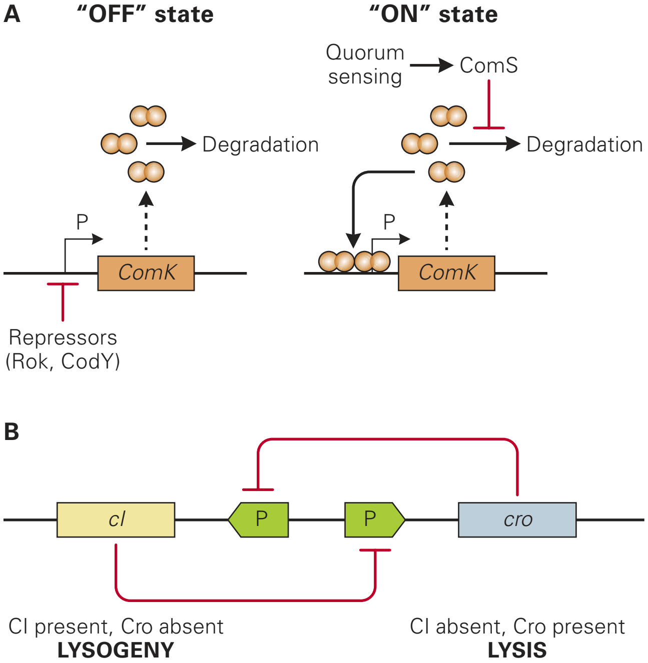


Figure 14-11. Examples of bistable switches. (A) Development of DNA competence in *Bacillus subtilis* is regulated by levels of ComK, which activates the transcription of genes required for DNA uptake. ComK also activates its own gene and forms a positive autoregulatory loop. In the "OFF" state, ComK expression is negatively regulated by several repressors, including Rok and CodY. Additionally, any leaky expression of ComK is readily degraded. In the "ON" state, quorum sensing leads to the synthesis of ComS, which in turn protects ComK from degradation. As ComK accumulates, it outcompetes repressors for binding to its own promoter, further activating expression of ComK. **(B)** The lysis/lysogeny decision of bacteriophage λ is a bistable switch that arises from a double-negative feedback loop of two mutually repressing regulatory proteins, CI and Cro. At the onset of infection, both repressors are produced, and each competes for binding to the promoter (P) of the gene for the other protein, thereby repressing its expression. If Cro binds first and represses expression of *cI*, then more Cro will be made and λ will lyse the bacterial cell. If CI binds first to repress expression of *cro*, then more CI will be made and λ will lysogenize the bacterial cell.

In addition to the gene rearrangements (deletions, insertions, duplications, inversions) discussed in chapter 7, a form of phase variation can also be achieved through gene regulatory networks, called **bistable switches**. These regulatory mechanisms control the transition between two phenotypic states, such that within a given bacterial population, there will be some individuals in each state. For example, as *Bacillus subtilis* enters stationary phase, about 10% of the cells become competent for genetic transformation—that is, one tenth of the population express the machinery required for DNA uptake. In *B. subtilis*, competence is controlled by the master regulator ComK, which is required to drive expression of DNA transport genes. The critical component of the system that makes it a bistable switch is that ComK also drives its own expression (Figure 14-11A). During exponential growth, competence is maintained in the "OFF" state as transcriptional repressors prevent ComK expression, and any ComK protein that is produced is quickly degraded. As the population enters stationary phase, quorum-sensing signals (which are discussed later in this chapter) trigger production of the ComS protein, which protects ComK from degradation. Without this degradation, any ComK protein produced by leaky expression is free to accumulate. Once ComK levels in a given cell rise past a critical threshold, the ComK in that cell starts driving its own expression, effectively overriding its transcriptional repression and resulting in the production of even more ComK. This positive feedback loop locks that cell's competence into the "ON" state. The frequency at which cells can reach this critical ComK threshold determines what fraction of the population will be in the "ON" state.

In addition to positive feedback loops, bistable switches can also arise from mutually repressing repressors. The prototypical example of such a system is the regulation controlling lysis and lysogeny of bacteriophage λ (Figure 14-11B). In this system, two transcriptional repressors, CI and Cro, mutually repress each other. That is, CI blocks the expression of Cro and Cro blocks the expression of CI. The ultimate cellular state is determined by which repressor can bind to the promoter first. If CI wins out, then the *cro* gene will be repressed, and the bacteriophage will lysogenize into the bacterial chromosome. However, if Cro wins out, then the *cI* gene will be repressed, and the bacteriophage will lyse the cell.

Similar bistable switches control a number of other bacterial processes, including sporulation, biofilm formation, motility, and virulence gene expression. These switches effectively force the bacterial population to always express multiple different phenotypes, thereby priming the population to respond to new external stimuli.

Hypermutability, Intragenomic Recombination, and Positive Selection

With the advent of rapid, inexpensive, next-generation DNA sequencing technologies, an increasingly large number of bacterial genomes have been fully sequenced. Sequence comparisons of related strains of the same bacterial species have revealed that bacterial genomes are constantly subject to numerous genomic rearrangements (e.g., deletions, insertions, inversions, duplications) that generate variability in the genomes (referred to as **genomic plasticity**) of individuals within a bacterial population. Like phase variation discussed in the previous section, this variability creates phenotypic diversity within the bacterial population, priming the population as a whole for adaptation to new selective environmental pressures.

One example of genomic plasticity in action is *Helicobacter pylori*, which lives in the harsh, low-pH environment of the stomach. Isolates of *H. pylori* from different individuals are highly diverse, much more so than would be expected for a bacterium that lives in an environment where there are little to no cocolonizing bacteria of different species through which horizontal gene transfer could occur. Some strains of *H. pylori* are **hypermutable** (i.e., have higher rates of mutations), but even this does not fully explain the high level of diversity observed. Rather, evidence points to the presence of numerous nonrandom, repetitive, direct-repeat sequences throughout the *H. pylori* genome, particularly within virulence-associated loci. These direct-repeat sequences generate the observed genomic plasticity by serving as sites for intragenomic recombination and chromosomal rearrangements.

One major class of intragenomic recombination mediated by direct-repeat sequences are **gene duplication and amplification (GDA)** events, which result in multiple copies of a given gene. GDA events are an important mechanism for adaptation to environmental stresses, particularly to antibiotics. They can result in an increased production of antibiotic-modifying enzymes, drug efflux pumps, or even the molecular targets of the antibiotics. In addition to changes in the genes themselves, GDA events can also occur in the promoter regions, ribosome-binding sites, or other transcriptional or translational regulatory elements, thereby causing antibiotic resistance-conferring genes to become constitutively active.

GDA events do place an increased metabolic burden on the bacteria, so most bacteria do not maintain spontaneous GDA events that occur from time to time. However, exposure to environmental stressors, such as antibiotics, applies a strong selective pressure for GDA events to persist, since the acquired resistances outweigh the fitness disadvantage of duplication of regions of the chromosome. Furthermore, GDA events can also drive the evolution of more efficient antibiotic resistance genes. By increasing the number of copies of a given resistance gene per cell, these events increase the likelihood of point mutations occurring in any one of them that improves its efficiency. The mechanisms of antibiotic resistance will be covered in further detail in chapter 16.

Coordinate Virulence Regulation

Bacterial pathogens are highly adapted to the hosts that they infect with specific genetically programmed strategies for overcoming host defenses, establishing colonization, multiplying, and ultimately disseminating to new host organisms. All of these processes are controlled by separate sets of genes whose regulation must be properly coordinated with each other, such that they are all expressed at the correct time and in response to the correct external stimuli. This **coordinate regulation of virulence** is typically

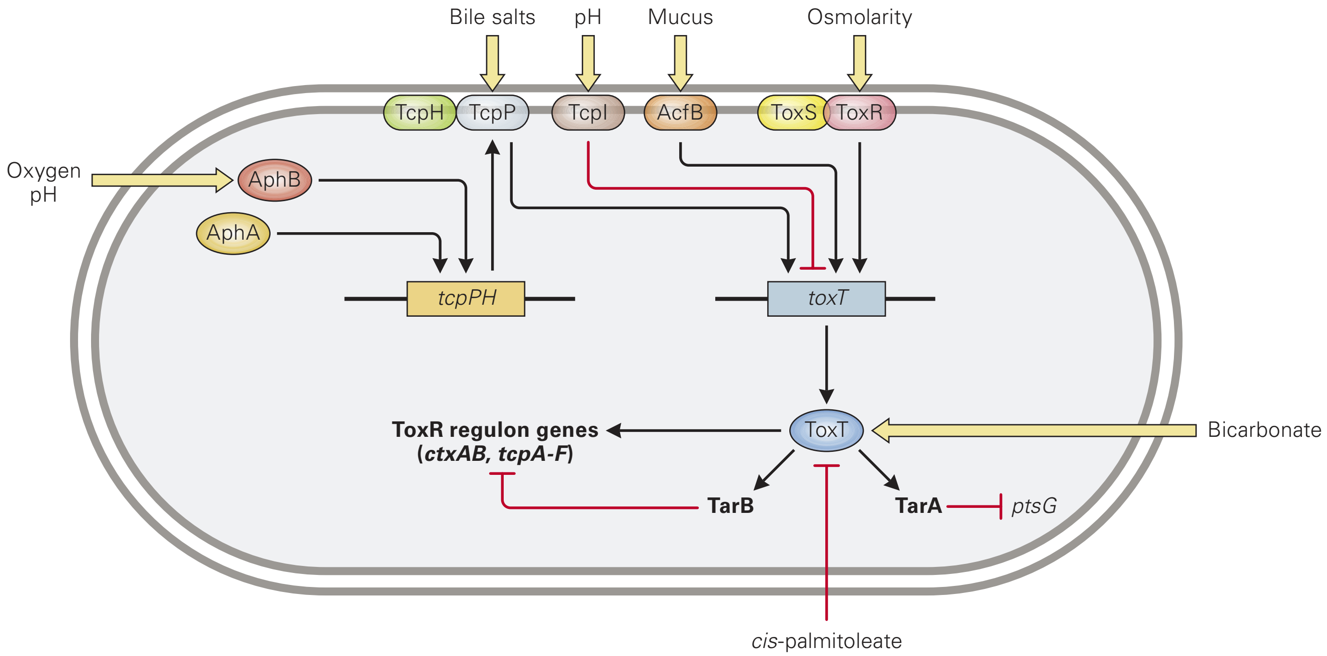


Figure 14-12. ToxT regulatory network in *Vibrio cholerae*. The global regulator ToxT constitutes a regulatory cascade that controls the transcription of virulence genes in *V. cholerae*. Environmental signals act through various sensor regulatory systems (ToxRS, TcpPH, TcpI, AcfB, AphAB) to regulate transcription of *toxT*. ToxT activates transcription of virulence genes, including that of cholera toxin (*ctxAB*) and the toxin coregulated pilus (*tcpA-F*), as well as regulatory sRNAs, TarA and TarB, which act as repressors of virulence genes. TarA interacts with Hfq and represses *ptsG*, which encodes a major glucose transporter in *V. cholerae*. TarB represses *ctxAB* and *tcpA-F*. Adapted from Peterson KM, Gellings PS. 2018. *Pathog Dis* **76(1)**, with permission.

maintained by complex regulatory networks involving multiple transcription factors and sensory mechanisms, many of which we have covered in the first half of this chapter.

Vibrio cholerae is the causative agent of the diarrheal disease cholera. Regulation of *V. cholerae* virulence genes at the transcriptional level revolves around activation of the global regulator ToxT in response to a number of different environmental signals (Figure 14-12). ToxT is a transcriptional activator that controls several sets of different virulence genes, including the *ctxAB* operon (encoding cholera toxin), the *tcp* operon (encoding toxin coregulated pilus), *acf* (accessory colonization factor), and as many as 20 other virulence genes collectively referred to as *tag* (ToxT-activated genes). More recently, it has been discovered that in some strains of *V. cholerae*, ToxT also controls a pair of regulatory sRNAs, TarA and TarB (ToxT-activated RNAs), which control additional cellular processes, including chemotaxis and motility. ToxT expression depends on a variety of different environmental signals, including pH, oxygen levels, nitric oxide, osmolarity, temperature, catabolite levels, mucus, and bile salts, all of which are sensed by a cascade of different regulators, several of which are summarized in Figure 14-12. Ultimately, when *V. cholerae* senses the right set of environmental signals (i.e., when the bacterium senses that it is in the host small intestine), ToxT is expressed, unlocking the expression of downstream virulence genes. Although we focused on regulatory networks in *V. cholerae*, it should be noted that nearly every pathogen has its own unique, complex regulatory network controlling its virulence.

Quorum Sensing

For a long time, bacteria were thought to exist as individual cells that acted independently from one another. We now know that bacteria are able to coordinate their activities as a group through small diffusible signaling molecules called **autoinducers**, which are usually produced under positive regulatory feedback control. At low cell densities, the concentration of these signaling molecules will be low, but as the cell density rises, these molecules accumulate to higher concentrations. Bacteria have developed ways of sensing the concentration of these molecules as a proxy for sensing the surrounding bacterial population density. This cell-cell communication is called **quorum sensing** and involves the direct or indirect activation of a cognate receptor protein by the autoinducer, which in turn directly or indirectly upregulates or downregulates a specific set of genes. By acting as a group or **quorum**, individuals are able to benefit from the activity of the entire group.

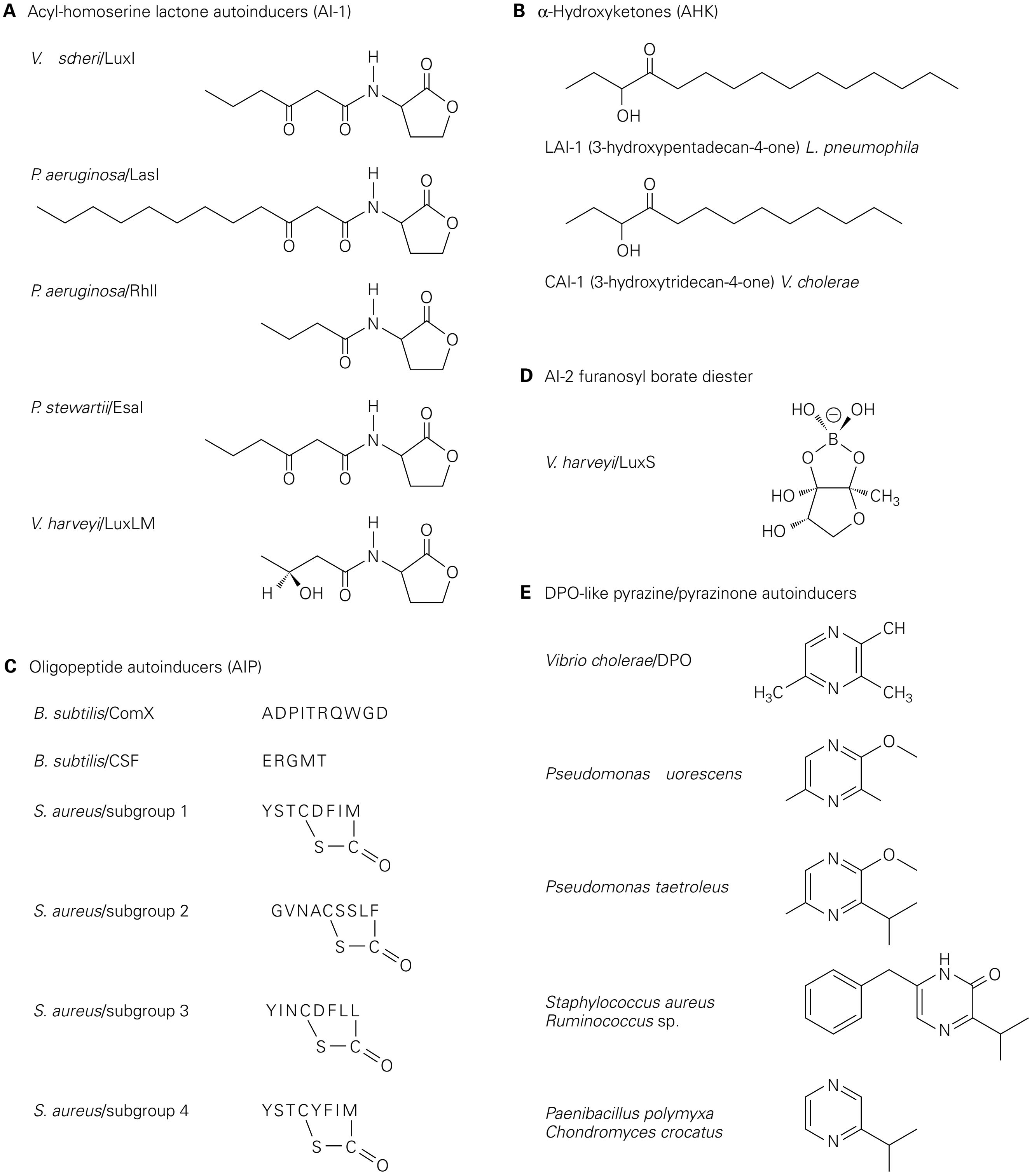
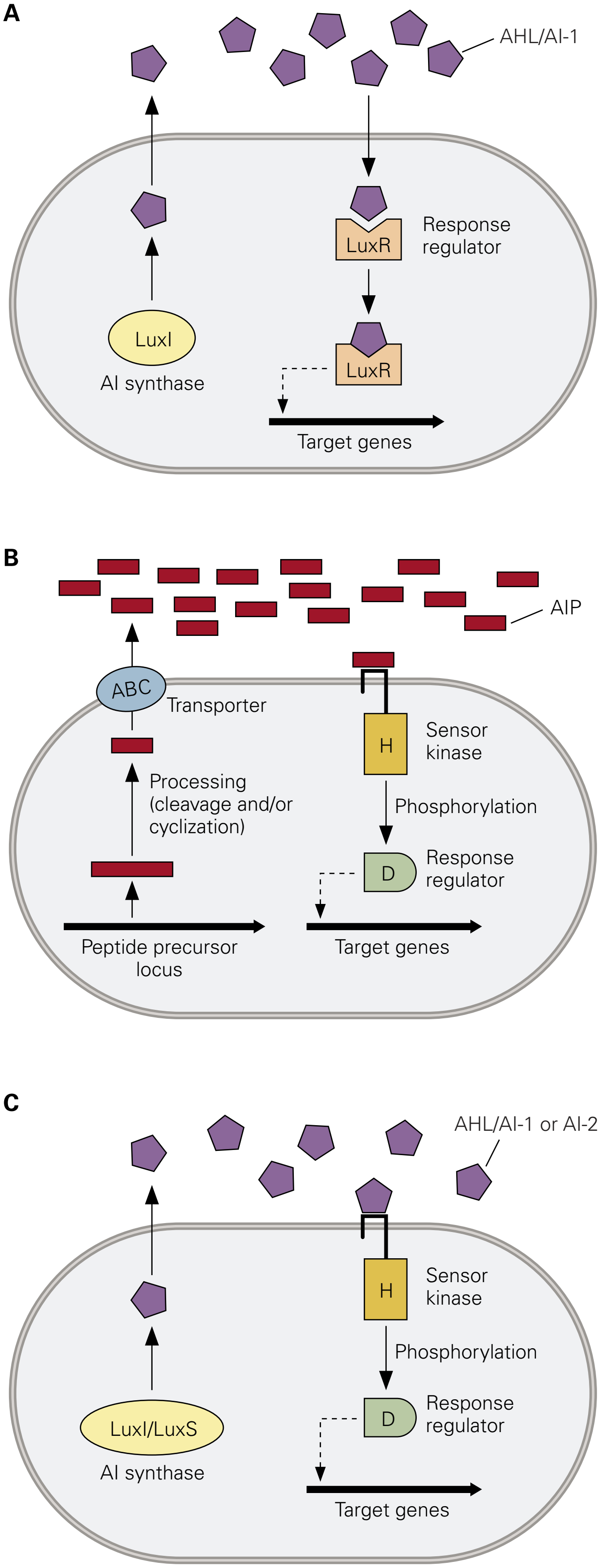

A Acyl-homoserine lactone autoinducers (AI-1)
V. fischeri/LuxI
P. aeruginosa/LasI
P. aeruginosa/RhlI
P. stewartii/EsaI
V. harveyi/LuxLM
B α-Hydroxyketones (AHK)
LAI-1 (3-hydroxypentadecan-4-one) L. pneumophila
CAI-1 (3-hydroxytridecan-4-one) V. cholerae
C Oligopeptide autoinducers (AIP)
B. subtilis/ComX
ADPITRQWGD
B. subtilis/CSF
ERGMT
S. aureus/subgroup 1
YSTCDFIM
S. aureus/subgroup 2
GVNACSSLF
S. aureus/subgroup 3
YINCDFLL
S. aureus/subgroup 4
YSTCYFIM
D AI-2 furanosyl borate diester
V. harveyi/LuxS
E DPO-like pyrazine/pyrazinone autoinducers
Vibrio cholerae/DPO
Pseudomonas fluorescens
Pseudomonas taetroleus
Staphylococcus aureus
Ruminococcus sp.
Paenibacillus polymyxa
Chondromyces crocatus

Figure 14-13. Quorum-sensing autoinducers. The quorum-sensing signaling molecules used in Gram-negative bacteria differ from those of Gram-positive bacteria. Shown are examples of autoinducers from different Gram-negative and Gram-positive bacteria. Also indicated are the names of corresponding proteins responsible for their biosynthesis. **(A)** Most Gram-negative bacteria use acyl-homoserine lactone (AHL) autoinducers. **(B)** α-hydroxyketones (AHKs) of *Legionella pneumophila* (LAI-1) and *Vibrio cholerae* (CAI-1). **(C)** Gram-positive bacteria use posttranslationally modified oligopeptides as autoinducers. Different *Staphylococcus aureus* subspecies produce different autoinducer peptides (e.g., subgroups 1-4) that can interfere with each other. **(D)** Furanosyl borate diester (autoinducer-2, AI-2), found in both Gram-negative and Gram-positive bacteria. **(E)** 3,5-Dimethylpyrazin-2-ol (DPO), produced by *V. cholerae* and other pyrazine and pyrazinone autoinducers (AIs), found in Gram-negative (*Pseudomonas fluorescens, Pseudomonas taetroleus*) and Gram-positive (*Staphylococcus aureus, Ruminococcus* sp., *Paenibacillus polymyxa, Chondromyces crocatus*) bacteria.


A
AHL/AI-1
LuxR
Response
regulator
LuxR
LuxI
AI synthase
Target genes
B
AIP
ABC
Transporter
H
Sensor
kinase
Processing
(cleavage and/or
cyclization)
Phosphorylation
D
Response
regulator
Peptide precursor
locus
Target genes
C
AHL/AI-1 or AI-2
H
Sensor
kinase
Phosphorylation
LuxI/LuxS
AI synthase
D
Response
regulator
Target genes

Quorum-sensing behavior has implications for many pathogens, where the outcome of the interaction between host and bacterium is strongly affected by the bacterial population size. By coupling the production of virulence factors with bacterial population density, many pathogens are able to limit the extent to which their mammalian host is able to respond to attacks. Put another way, by the time the host realizes that there is a problem, the bacterial population will have already grown large enough to resist many host defenses. Being able to sense bacterial population density also enables an invading pathogen to know when a host site has become saturated and that it is time to spread to a new site or a new host by expressing certain specialized toxin and/or motility genes. Quorum sensing allows for coordinately regulated expression of a wide range of cellular processes, including bioluminescence, sporulation, and DNA conjugation, and different virulence factors, including toxins, adhesins, flagella, capsules, siderophores, antibiotic resistance, biofilm formation, and secretion systems.

Each species of Gram-negative or Gram-positive bacteria uses its own distinct set of quorum-sensing autoinducers, receptors, and signaling circuitry. It is beyond the scope of this book to cover them all in detail. Instead, we provide here a few generalized mechanisms for Gram-negative and Gram-positive bacteria followed by some well-studied examples.

Signaling molecules. Most Gram-negative bacteria use **N-acyl homoserine lactones (AHLs)** (Figure 14-13A) as intraspecies quorum-sensing signals although other signaling molecules, such as the **α-hydroxyketones (AHKs)** (Figure 14-13B), have also been discovered in some bacteria. The AHL and AHK autoinducers are small, relatively hydrophobic molecules that readily diffuse across bacterial cell membranes (Figure 14-14). AHL is synthesized from S-adenosyl methionine (SAM) and a fatty acid precursor, acyl-ACP (acyl carrier protein) (Figure 14-15A). Similarly, AHK is derived from the condensation of a fatty acyl-CoA with an amino acid molecule such as 2-amino-butyrate through a pyridoxal phosphate (PLP)-dependent reaction, followed by conversion of the amino

Figure 14-14. Quorum-sensing systems of Gram-negative and Gram-positive bacteria. (A) Prototypical autoinducer quorum-sensing system utilized for AHL and AHK (AI-1) signaling molecules in Gram-negative bacteria involves binding of the autoinducer to a cytoplasmic response regulator protein that is not part of a TCS. The LuxIR system of *Vibrio fischeri* is shown as an example. **(B)** Autoinducer oligopeptide (AIP) TCS quorum-sensing system of Gram-positive bacteria. **(C)** The hybrid autoinducer TCS quorum-sensing systems utilized for AHL/AI-1 and AI-2 signaling molecules in Gram-negative bacteria and for AI-2 in Gram-positive bacteria.

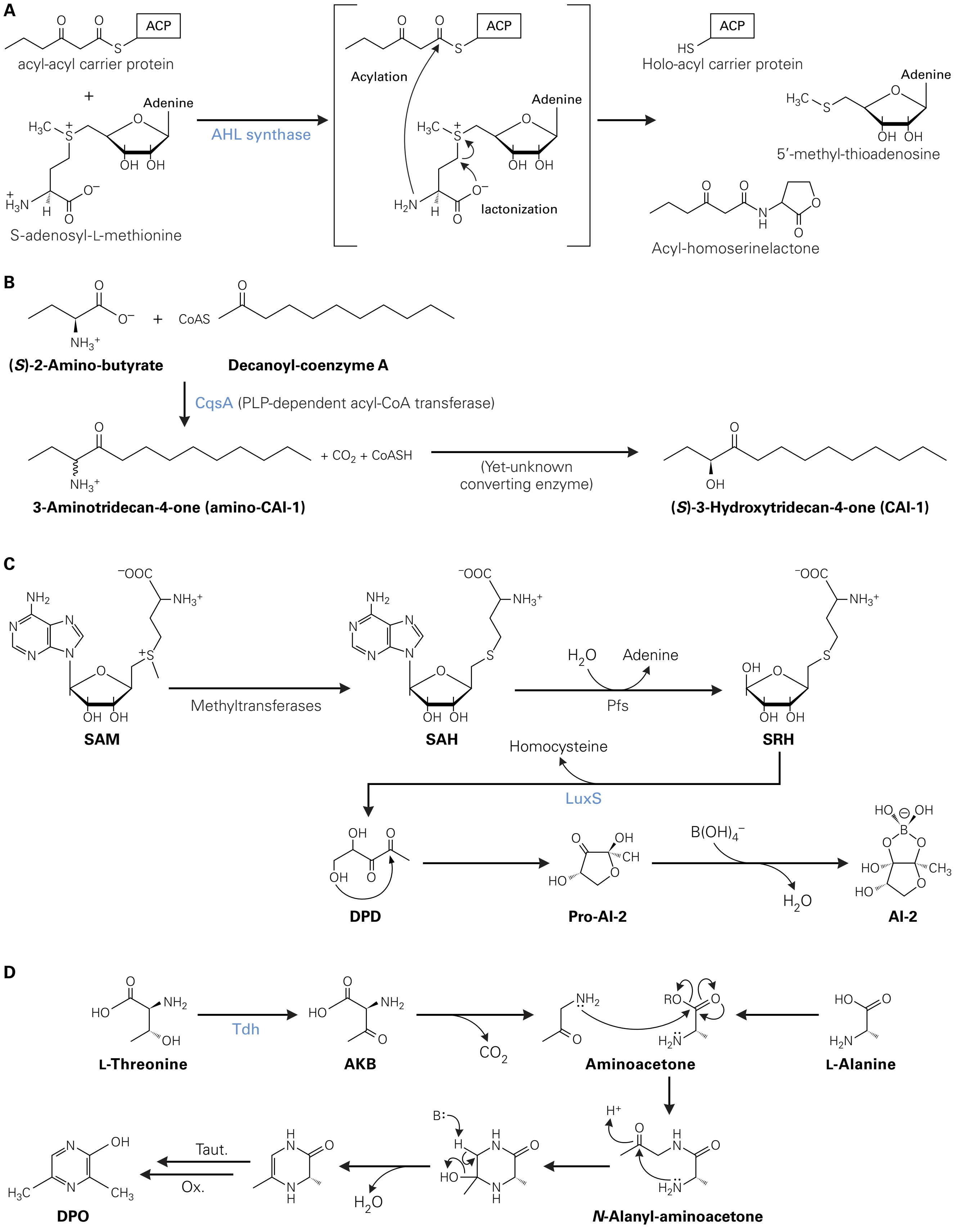

A
ACP
acyl-acyl carrier protein
+
Adenine
S-adenosyl-L-methionine
AHL synthase
Acylation
lactonization
Holo-acyl carrier protein
5′-methyl-thioadenosine
Acyl-homoserinelactone
B
(S)-2-Amino-butyrate
CoAS
Decanoyl-coenzyme A
CqsA (PLP-dependent acyl-CoA transferase)
+ CO2 + CoASH
3-Aminotridecan-4-one (amino-CAI-1)
(Yet-unknown converting enzyme)
(S)-3-Hydroxytridecan-4-one (CAI-1)
C
SAM
Methyltransferases
SAH
H2O
Adenine
Pfs
SRH
Homocysteine
LuxS
DPD
Pro-AI-2
B(OH)4−
H2O
AI-2
D
L-Threonine
Tdh
AKB
CO2
Aminoacetone
L-Alanine
N-Alanyl-aminoacetone
B:
H2O
Taut.
Ox.
DPO

Figure 14-15. **Biosynthesis of autoinducers AHL, AI-2, AHK, and DPO. (A)** Different AHLs (AI-1s) are derived from S-adenosyl methionine (SAM) condensed to various side chains on an acyl-carrier protein. **(B)** AHKs, such as CAI-1 from *Vibrio cholerae*, are derived from the reaction of a fatty acyl-CoA with 2-amino-butyrate, through the action of a pyridoxal phosphate-dependent acyl-CoA transferase, followed by conversion of the amino group into a hydroxyl group by an as-yet-unidentified enzyme. **(C)** Like AHL, AI-2 is derived from S-adenosyl methionine (SAM) during its normal catabolic degradation pathway. The last enzymatic step involves LuxS, which produces DPD. Conversion to AI-2 occurs nonenzymatically in the presence of borate. **(D)** Proposed biosynthesis of DPO from *V. cholerae*. Threonine dehydrogenase (Tdh) catalyzes the oxidation of threonine (Thr) to 2-amino-3-ketobutyric acid (AKB), which spontaneously decarboxylates to aminoacetone. Alanine (Ala) is condensed with aminoacetone to yield *N*-alanyl-aminoacetone, followed by cyclization, dehydration, tautomerization (Taut.), and oxidation (Ox.) to yield DPO by as-yet-unidentified enzyme(s).

group into a hydroxyl group (Figure 14-15B). Binding of the AHL or AHK ligand to its respective cytoplasmic response regulator protein results in dimerization of the regulator, which then binds to the promoter region of target genes and thereby modulates the upregulation or downregulation of those genes (Figure 14-14A). In addition, some AHL ligands bind to the extracellular receptor domain of a histidine kinase (Figure 14-14C), which is a member of a TCS (see the section on TCSs earlier in this chapter).

By contrast, Gram-positive bacteria use small posttranslationally modified peptides as intraspecies autoinducers (Figure 14-13C). These **autoinducing peptides (AIPs)** are too hydrophilic to cross membranes on their own and therefore remain in the extracellular medium once transported out of the bacterial cell (Figure 14-14B). AIPs are expressed as larger precursor peptides that are then processed into smaller, sometimes cyclic thiolactone-containing peptides that are transported cross the bacterial cell membrane (Figure 14-16). Sensing of the AIP occurs through the binding of the AIP to its cognate receptor that is a transmembrane protein located on the surface of the bacteria. The receptor is the histidine kinase of a TCS (Figure 14-14B). Binding of the AIP to the sensor kinase induces autophosphorylation of the kinase on a conserved histidine residue, from which the phosphoryl group is transferred to an aspartate residue of the cognate response regulator. The phosphorylated response regulator in turn binds to the promoter regions of target genes and activates or represses their transcription.

A different type of quorum-sensing signaling molecule is used for interspecies cell-cell communication. This **autoinducer-2 (AI-2)** is a small furanosyl borate diester compound (Figure 14-13D) that is produced during the catabolic degradation pathway of S-adenosyl methionine (SAM) and requires three enzymatic steps (Figure 14-15C), the last one involving LuxS, which converts S-ribosylhomocysteine (SRH) into 4,5-dihydroxy-2,3-pentanedione (DPD). The DPD product then nonenzymatically cyclizes and converts into AI-2 in the presence of borate. The advantage of using a molecule that is the product of a metabolic process common to all bacteria is that its production is a clear indicator of the presence of other bacteria but is independent of the type of bacteria that produce it. Detecting the concentration of such a product allows a bacterium to sense how many bacteria are competing with it for colonization and

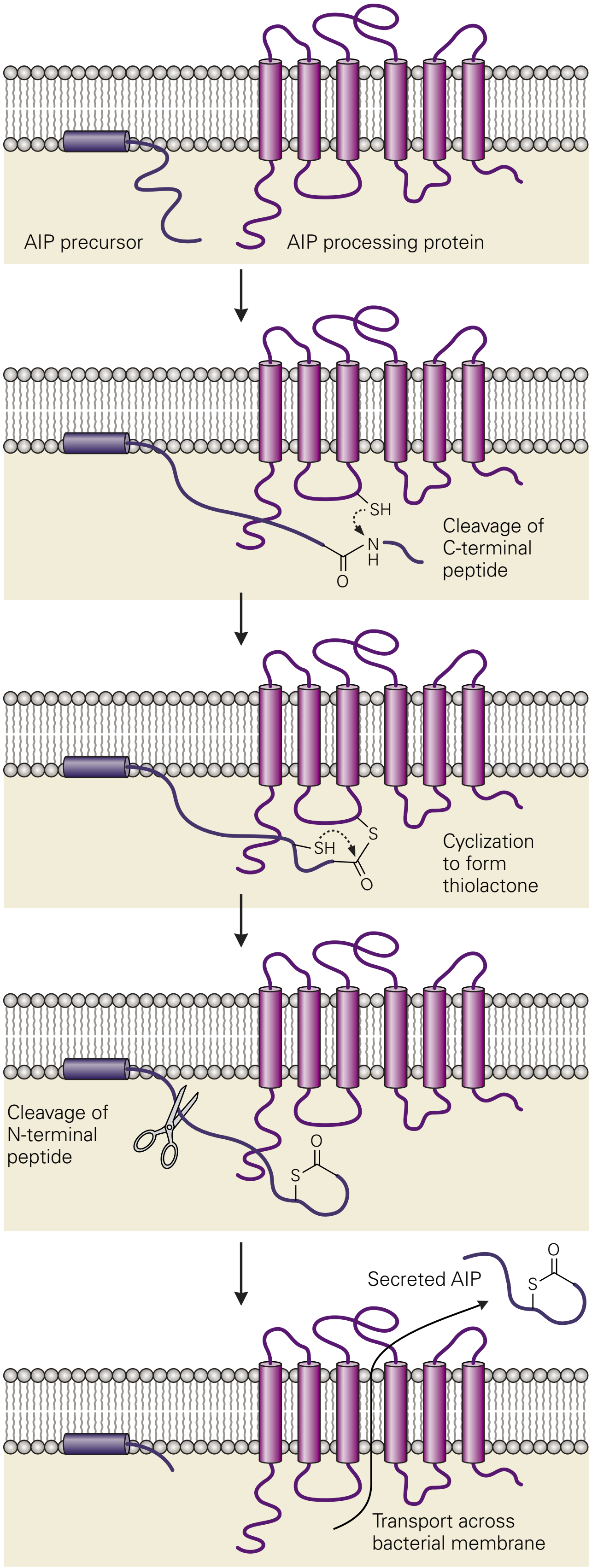

AIP precursor
AIP processing protein
SH
N
H
O
Cleavage of C-terminal peptide
S
SH
O
Cyclization to form thiolactone
Cleavage of N-terminal peptide
O
S
Secreted AIP
O
S
Transport across bacterial membrane

Figure 14-16. Processing of autoinducing peptides (AIPs). In Gram-positive bacteria, the quorum-sensing autoinducing peptide (AIP) is derived from a larger precursor peptide that is posttranslationally modified into a smaller peptide by a transmembrane processing protein that mediates the catalytic cleavage and transport of the AIP across the bacterial membrane. During the process the peptide is often cyclized into a thiolactone using the thiol side chain of a cysteine residue.

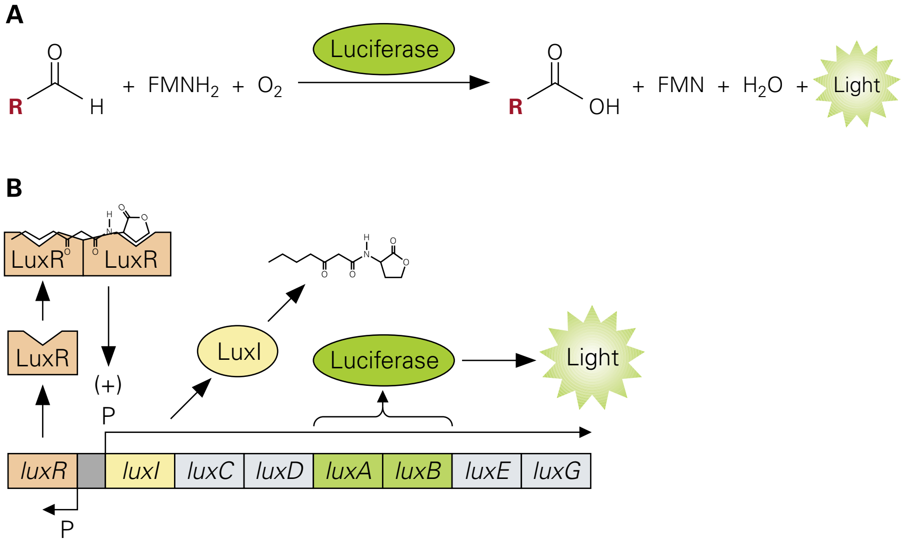


Figure 14-17. Luciferase-catalyzed bioluminescence. In the Lux system of *Vibrio fischeri* (see Figure 14-14A), bioluminescence is generated through the action of two sets of proteins, one responsible for the production of the luciferase (the proteins encoded by *luxCDABEG*) and the other responsible for regulating the Lux system through quorum sensing (LuxR and LuxI). **(A)** Luciferase generates blue-green light through the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde (RCHO) in the presence of oxygen. **(B)** Organization of the *lux* operon. The AHL (AI-1) produced by LuxI binds LuxR to positively regulate transcription of the other *lux* genes.

growth. The receptor of AI-2 is a histidine kinase of a TCS (Figure 14-14C).

Recently, a new type of autoinducer-receptor signaling pair involved in the regulation of biofilm formation and virulence has been found in both Gram-positive and Gram-negative bacteria. In *V. cholerae*, this new autoinducer is called 3,5-dimethylpyrazin-2-ol (DPO) (Figure 14-13E) and binds to its response regulator, the transcription factor VqmA, and turns on expression of the regulatory sRNA VqmR, which in turn represses biofilm formation and toxin production. Formation of DPO occurs as a result of condensation of alanine with aminoacetone, a product of the threonine degradation pathway common to many bacteria (Figure 14-15D). The enzyme threonine dehydrogenase (Tdh) initiates the process by catalyzing the oxidation of threonine. After the condensation reaction to form N-alanyl-aminoacetone, a series of additional reactions catalyzed by an as-yet-unknown enzyme(s) yields the pyrazine DPO.

The Lux system of Vibrio fischeri. Quorum sensing in Gram-negative bacteria was first described in the bacterium *Vibrio fischeri*, a bioluminescent symbiont of several different marine animals, most notably the bobtail squid, *Euprymna scolopes*. The squid cultivate *V. fischeri* within a light organ. At night, light emitted from this organ is directed downward and serves to obfuscate the silhouette of the squid from the vision of predators swimming beneath the squid. At sunrise, the squid expels 90% to 95% of the bacterial population from its light organ, then burrows itself into the sand of the ocean floor as its bacterial population regrows over the course of the day. It is not known exactly why the squid expels its bacterial population, but it may be related to the *V. fischeri* needing to maintain a growing state in order to stably luminesce.

V. fischeri bioluminescence results from transcriptional activation of the bacterial Lux operon, *luxCDABEG*, where *luxA* and *luxB* encode the luciferase protein subunits and other *lux* genes encode proteins that form a biosynthetic enzyme complex, which synthesizes the long-chain aldehyde substrate that is oxidized by the luciferase to produce blue/green light (Figure 14-17A). There are several different tiers of regulation of the *lux* operon (Figure 14-17B). At the first level, LuxI is produced constitutively at low levels and is responsible for producing the acyl-homoserine lactone, autoinducer-1 (AI-1). In *V. fischeri*, this molecule is 3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL). When the cell density of the bacterial culture increases, the AHL reaches a threshold concentration that causes it to bind and activate the LuxR response regulator protein through dimerization. The AHL-bound LuxR dimer then binds to the promoter region of the *lux* operon and positively regulates both the *luxCDABEG* genes responsible for luminescence, as well as the *luxI* gene, resulting in the production of even more AHL.

Layered onto the LuxIR quorum-sensing regulation are interconnected phosphorelay pathways (Figure 14-18). AinR and LuxPQ are sensor kinases that each respond to a

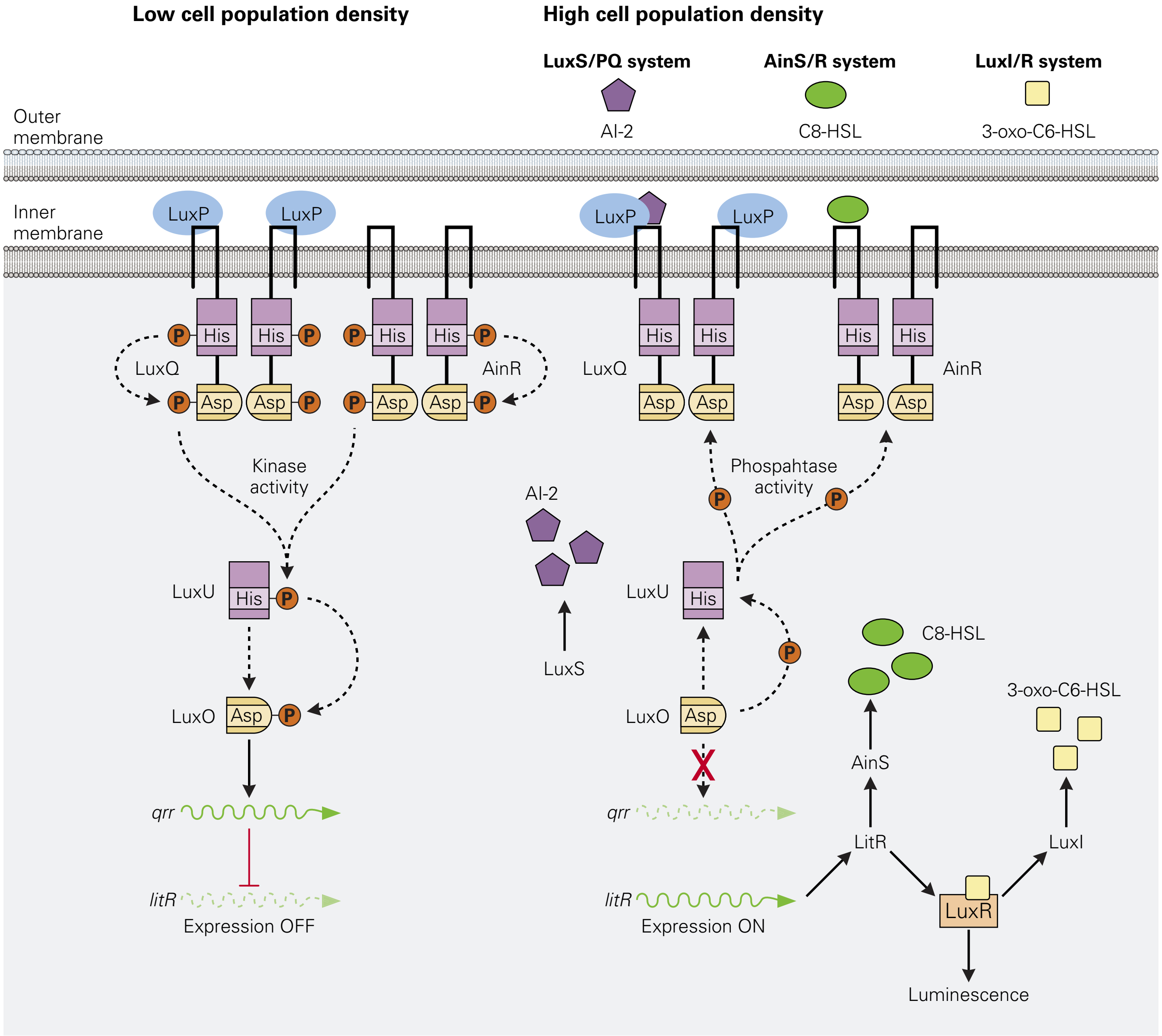

Low cell population density
High cell population density
LuxS/PQ system
AinS/R system
LuxI/R system
AI-2
C8-HSL
3-oxo-C6-HSL
Outer membrane
Inner membrane
LuxP
LuxP
LuxP
LuxP
His
Asp
P
LuxQ
AinR
LuxQ
AinR
Kinase activity
Phospahtase activity
AI-2
LuxS
LuxU
LuxU
LuxO
LuxO
C8-HSL
AinS
3-oxo-C6-HSL
qrr
qrr
litR
litR
Expression OFF
Expression ON
LitR
LuxI
LuxR
Luminescence

Figure 14-18. Quorum-sensing systems of *Vibrio fischeri*. *V. fischeri* has two AHL quorum-sensing systems, the hybrid AinSR system, and the LuxIR system, all of which work together in a sequential manner to regulate motility, colonization, and bioluminescence. Adapted from Milton DL. 2006. *Int J Med Microbiol* **296:**61–71, with permission.

different quorum-sensing signal molecule. AinR responds to octanoyl-homoserine lactone (C8-HSL) produced by AinS, while LuxPQ senses AI-2, produced by LuxS. In the absence of these quorum-sensing molecules at low cell densities, AinR and LuxPQ are active kinases, which autophosphorylate and transfer a phosphoryl group to the phosphotransfer protein, LuxU, which in turn phosphorylates LuxO. The phosphorylated LuxO (LuxO~P) response regulator activates the σ^{54}-containing RNA polymerase to increase expression of the regulatory sRNA gene, *qrr1*. The Qrr1 sRNA then inhibits translation of the LitR regulator, which is a direct transcriptional activator of the *luxR* gene. Hence, at low cell densities, luminescence is turned off. At high cell densities, this phosphotransfer pathway is reversed with AinR and LuxPQ serving as phosphatases to remove the phosphoryl group from LuxU and in turn from LuxO. When LuxO is dephosphorylated, Qrr1 levels decrease, allowing translation of LitR and thus expression of LuxR and increased luminescence. It should be noted that this paradigm of quorum sensing-dependent TCSs linked to sRNA-mediated translation inhibition of a key transcription activator is found in many pathogens, such as *V. cholerae*. In this case, sRNA inhibition of translation of the HapR transcriptional regulator leads to increased biofilm forma-

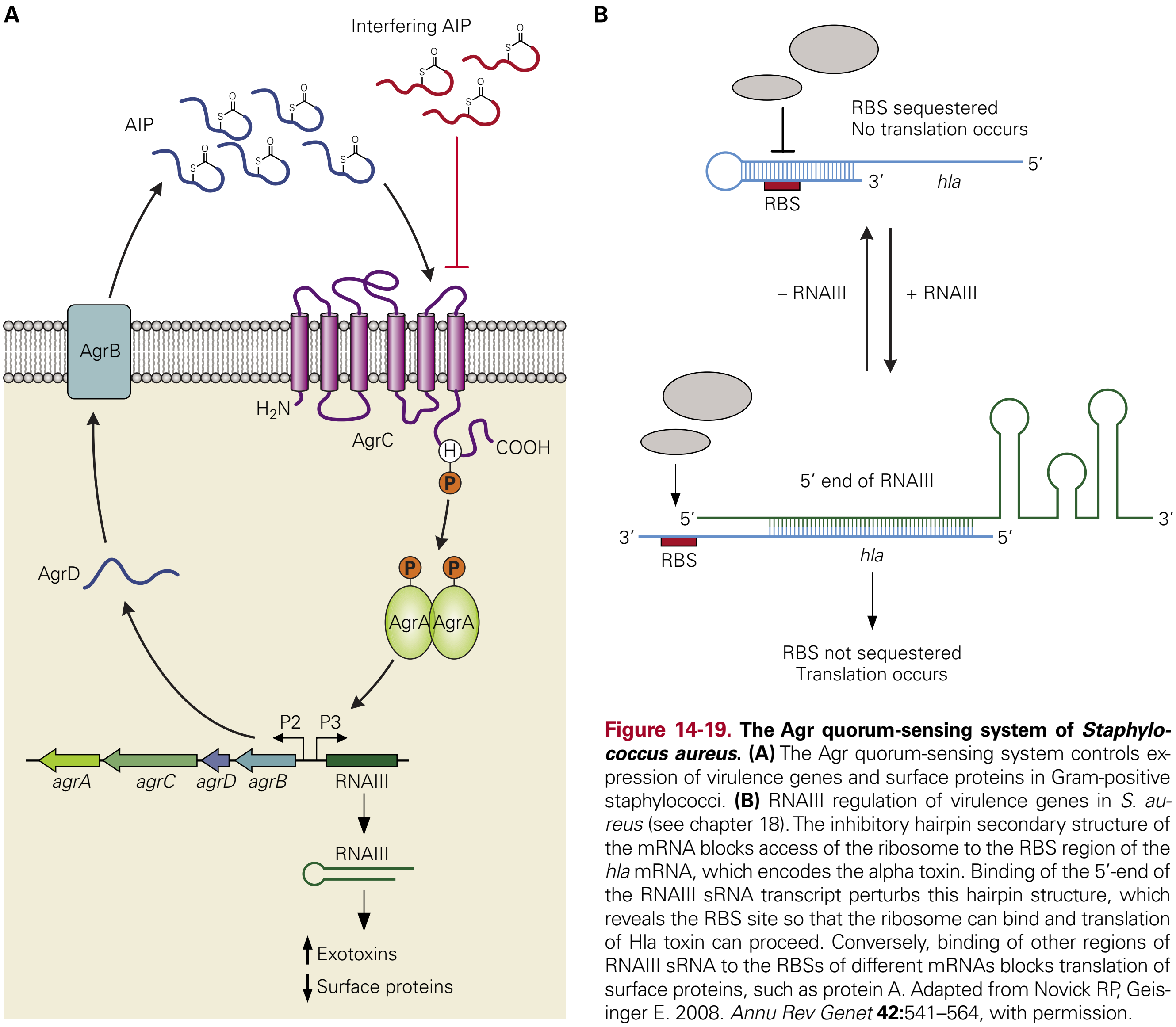


Figure 14-19. The Agr quorum-sensing system of *Staphylococcus aureus*. (A) The Agr quorum-sensing system controls expression of virulence genes and surface proteins in Gram-positive staphylococci. **(B)** RNAIII regulation of virulence genes in *S. aureus* (see chapter 18). The inhibitory hairpin secondary structure of the mRNA blocks access of the ribosome to the RBS region of the *hla* mRNA, which encodes the alpha toxin. Binding of the 5′-end of the RNAIII sRNA transcript perturbs this hairpin structure, which reveals the RBS site so that the ribosome can bind and translation of Hla toxin can proceed. Conversely, binding of other regions of RNAIII sRNA to the RBSs of different mRNAs blocks translation of surface proteins, such as protein A. Adapted from Novick RP, Geisinger E. 2008. *Annu Rev Genet* **42:**541–564, with permission.

tion and decreased virulence factor production at low cell densities. At high cell densities, quorum sensing reverses this expression pattern by reducing biofilm formation and increasing virulence factor expression.

***The Agr system of* S. aureus.** The Agr system in *S. aureus* was one of the first quorum-sensing systems elucidated in Gram-positive bacteria (Figure 14-19). The expression of most virulence factors, including toxins and numerous surface proteins, is globally controlled by the *agr* locus, which contains genes for a two-component signaling system (*agrAC*), for the biosynthesis and export of the activating ligand (*agrDB*), and for the regulatory sRNA (RNAIII). The *agrD* gene encodes the 46-amino-acid precursor peptide of the cyclic thiolactone-containing AIP that is processed and transported out of the cell by the transmembrane protein AgrB. The thiol group of this cysteine is used to form the cyclic thiolactone structure of the secreted AIP. The secreted AIP serves as the ligand for the transmembrane receptor AgrC, which is the sensor histidine kinase that transduces the signal through phosphorylation of its cognate response regulator AgrA. Phosphorylated AgrA activates transcription from the *agr* P2 and P3 promoters, which in turn upregulate the expression of the *agrACDB* genes from the P2 promoter in one direction and expression of the *agr* sRNA RNAIII through the P3 promoter in the opposite direction. RNAIII is the global regulator that upregulates many genes for virulence factors and secreted proteins, while downregulating genes for certain surface proteins.

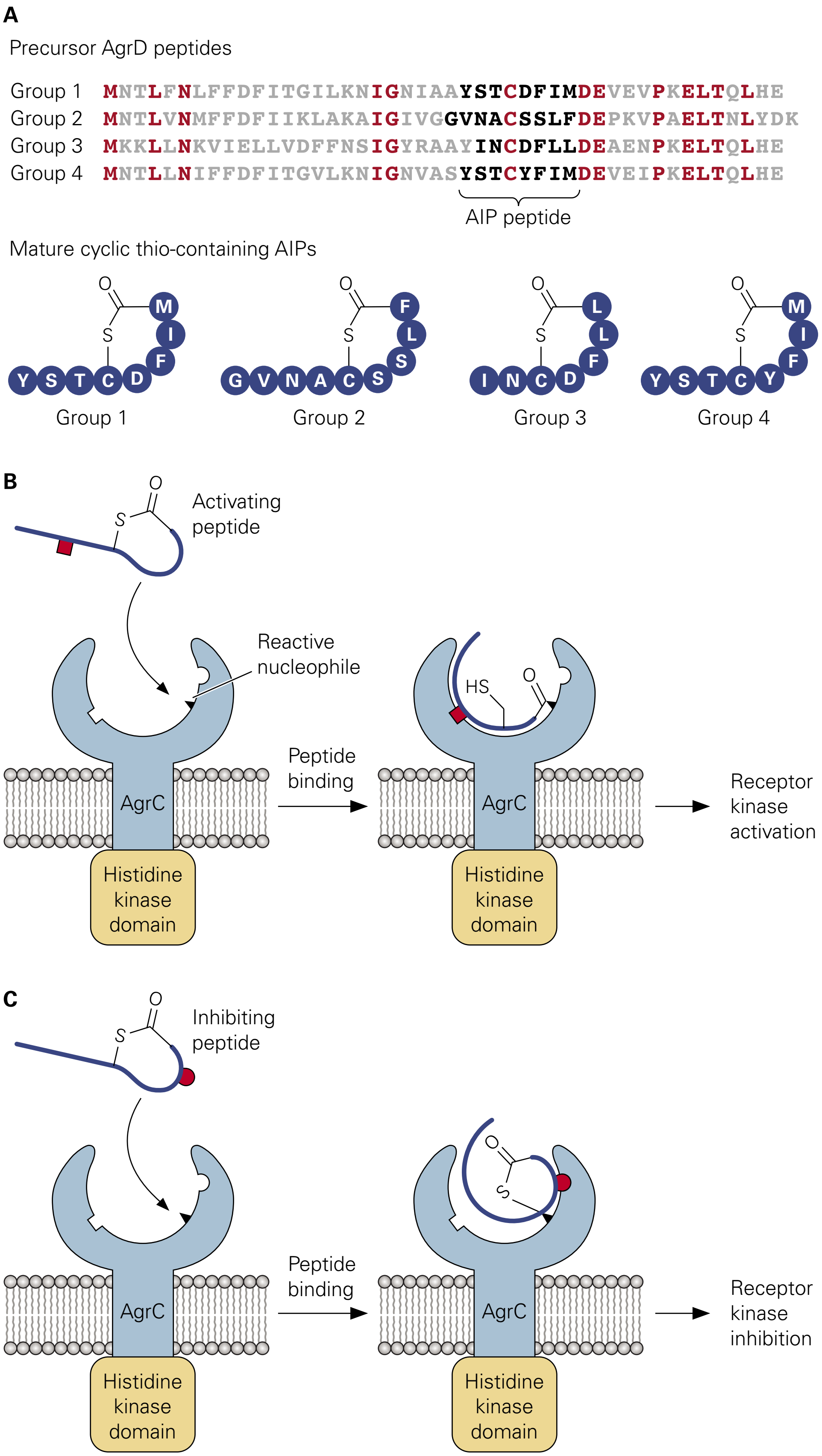

A
Precursor AgrD peptides
Group 1 MNTLFNLFFDFITGILKNIGNIAAYSTCDFIMDEVEVPKELTQLHE
Group 2 MNTLVNMFFDFIIKLAKAIGIVGGVNACSSLFDEPKVPAELTNLYDK
Group 3 MKKLLNKVIELLVDFFNSIGYRAAYINCDFLLDEAENPKELTQLHE
Group 4 MNTLLNIFFDFITGVLKNIGNVASYSTCYFIMDEVEIPKELTQLHE
AIP peptide
Mature cyclic thio-containing AIPs
O
S
Y S T C D F I M
Group 1
O
S
G V N A C S S L F
Group 2
O
S
I N C D F L L
Group 3
O
S
Y S T C Y F I M
Group 4
B
O
S
Activating peptide
Reactive nucleophile
AgrC
Histidine kinase domain
Peptide binding
HS
O
AgrC
Histidine kinase domain
Receptor kinase activation
C
O
S
Inhibiting peptide
AgrC
Histidine kinase domain
Peptide binding
O
S
AgrC
Histidine kinase domain
Receptor kinase inhibition

Figure 14-20. **Bacterial interference in *Staphylococci*.** **(A)** Sequences of precursor AgrD peptides from *S. aureus* groups 1-4. **(B)** Activation of AgrC receptor by cognate AIP ligand. **(C)** Inhibition of AgrC receptor by AIP ligand from a different staphylococcal group. Panels B and C adapted from Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW. 1999. *Proc Natl Acad Sci USA* **96:**1218–1223, with permission.

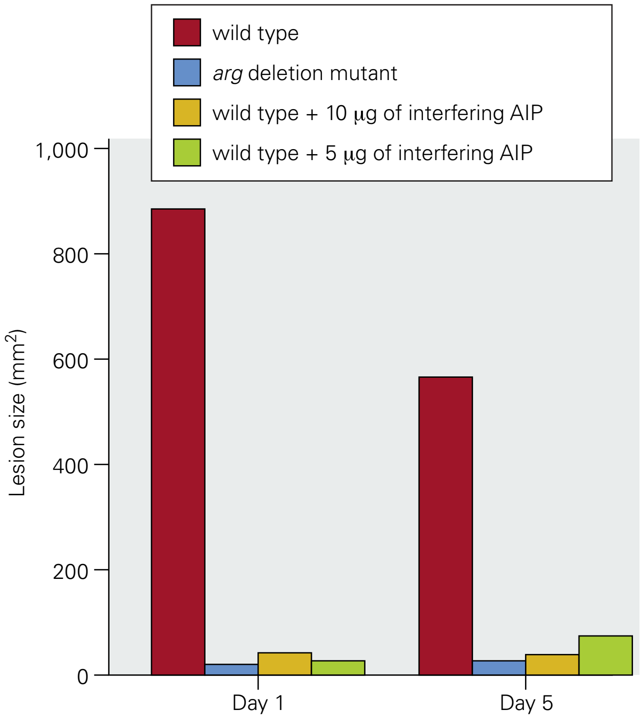


Figure 14-21. Experiment demonstrating control of staphylococcal infection through bacterial interference. Staphylococcal skin abscess formation in mice could be inhibited by treatment with a thiolactone-containing AIP that could compete with the wild-type AIP for binding to the AgrC receptor but could not induce signaling, which effectively blocked virulence in the inoculated wild-type (wt) bacteria. A mutant bacterium lacking the *agr* locus (denoted as *agr* deletion mutant) was used as a negative control. Size of the skin lesion caused by injection of the wild-type staphylococci or *agr* deletion mutant in the presence or absence of 5 or 10 µg of interfering AIP. Data from Mayville P, Ji G, Beavis R, Yang H, Goger M, Novick RP, Muir TW. 1999. *Proc Natl Acad Sci USA* **96:**1218–1223.

The mature AIPs from different staphylococcal species consist of seven to nine amino acids with a highly conserved cysteine at a position located five residues from the C-terminus (Figure 14-20). The *agr* genes show considerable sequence variation but can be divided into four distinct groups based on the specificity of the AIPs and their cognate histidine kinase receptors. Variations in the structures of the AIPs have compensatory changes in the sequences of the corresponding AgrB processor and AgrC receptor proteins at sites involved in protein-ligand interactions. AIPs from different staphylococcal species interfere with the signaling from each other, such that AIPs with one group activate the *agr* response within the same group, but inhibit *agr*-mediated regulation in strains producing different AIPs. It is thought that an AIP binds its cognate AgrC receptor in a different manner than it does a receptor from another group. This interesting mechanism of **bacterial interference** has been exploited for development of therapeutics against staphylococcal infections (Figure 14-21).

Chemotaxis

Thus far in this section, we have covered several different mechanisms by which bacteria are able to passively react to their environment. However, many bacteria are also capable of actively seeking out new, more favorable environments through regulation of their cellular motility machinery. In a process called **chemotaxis**, these bacteria are able to bias the direction of their movement toward regions with higher concentrations of certain chemical signals called **chemoattractants**. Conversely, bacteria can also bias their movement away from toxic compounds or conditions that act as **chemorepellents**. In general, bacteria are too small to accurately sense chemical gradients across the length of the cell. Instead, they detect chemical gradients continuously sensing changes in their local environment as they move. In the absence of any signals, a motile bacteria cell will spontaneously change its direction from time to time, analogous to a random walk (Figure 14-22A). However, if during the cell's movement it encounters more favorable conditions, the cell will maintain the direction of its movement for a longer period of time before spontaneously changing direction (Figure 14-22B). Over time, this behavior will result in a biased random walk-like movement toward the more favorable conditions. One critical aspect of this sensing mechanism is that the cell must not only detect chemical signals indicative of a favorable environment, but it must also be able to decide if a newly encountered local environment is better than the one it was in just a moment ago.

Different species of motile bacteria employ different mechanisms to sense chemical gradients and possess different flagellar machinery to drive their movement, but the best understood chemotaxis system is that of *E. coli*. *E. coli* cells each have five to eight peritrichous flagella. As was described in detail in chapter 11, when all of the flagella engage in the same counterclockwise rotation, the flagella will bundle together and cause the cell to undergo smooth swimming and travel in a straight line. However, if one or more of the flagella switch the direction of their rotation, the flagellar bundle will unwind causing the cell to tumble in place. When smooth swimming resumes by counterclockwise rotation of flagella, the cell then travels in a new random direction.

This direction switching is controlled through a phosphorelay system (Figure 14-22C). When phosphorylated, the CheY response regulator protein binds to the flagellar motor and switches the rotation from the default counterclockwise direction to the clockwise direction. CheY is phosphorylated by histidine kinase CheA. CheA functions analogously to the sensor kinases of two-component systems, except that CheA is not itself a receptor. Instead, it

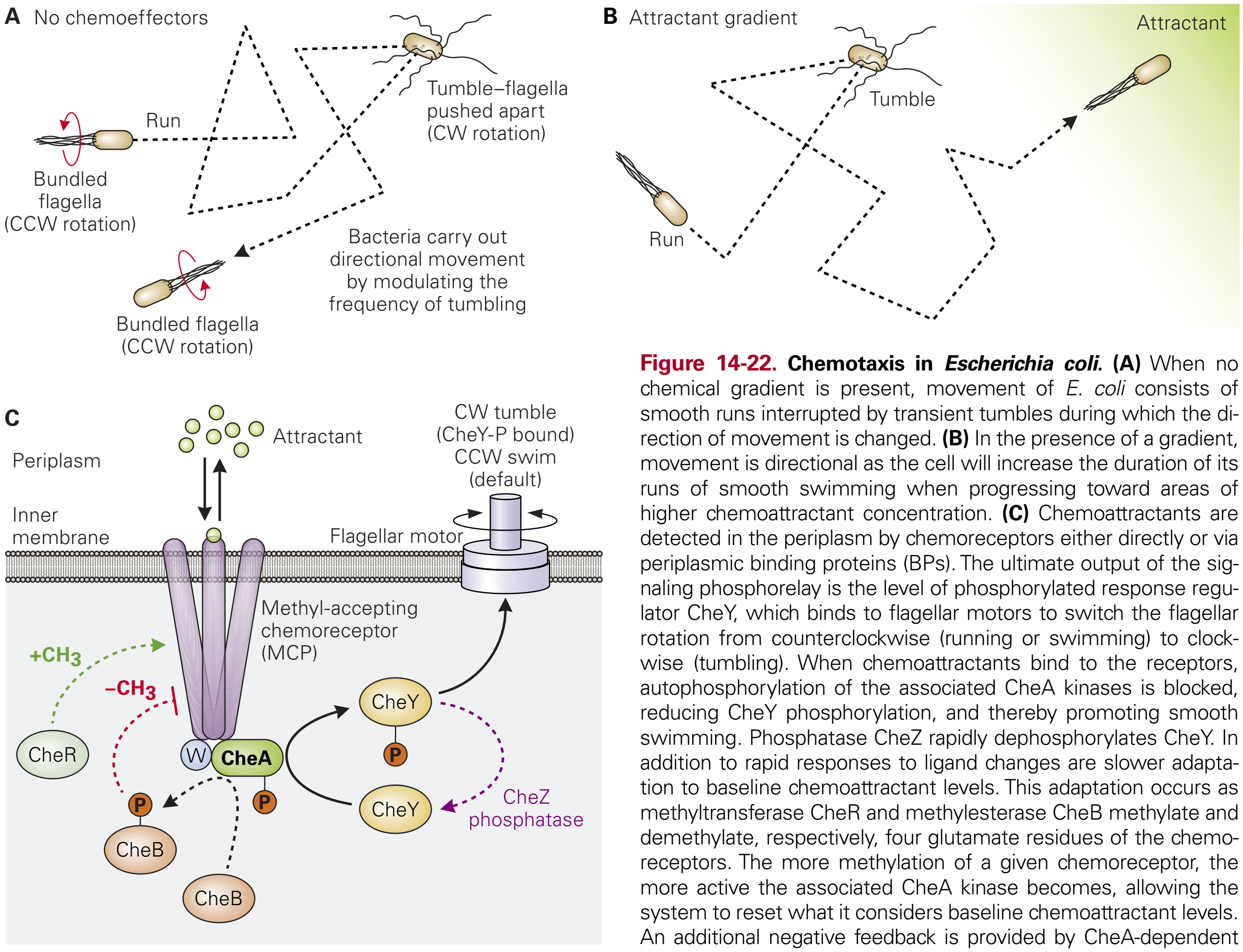

A No chemoeffectors
Run
Tumble–flagella pushed apart (CW rotation)
Bundled flagella (CCW rotation)
Bacteria carry out directional movement by modulating the frequency of tumbling
Bundled flagella (CCW rotation)
B Attractant gradient
Attractant
Tumble
Run
C
Periplasm
Attractant
CW tumble (CheY-P bound) CCW swim (default)
Inner membrane
Flagellar motor
Methyl-accepting chemoreceptor (MCP)
+CH3
–CH3
CheR
W
CheA
P
P
CheB
CheB
CheY
P
CheY
CheZ phosphatase

Figure 14-22. Chemotaxis in *Escherichia coli*. (A) When no chemical gradient is present, movement of *E. coli* consists of smooth runs interrupted by transient tumbles during which the direction of movement is changed. **(B)** In the presence of a gradient, movement is directional as the cell will increase the duration of its runs of smooth swimming when progressing toward areas of higher chemoattractant concentration. **(C)** Chemoattractants are detected in the periplasm by chemoreceptors either directly or via periplasmic binding proteins (BPs). The ultimate output of the signaling phosphorelay is the level of phosphorylated response regulator CheY, which binds to flagellar motors to switch the flagellar rotation from counterclockwise (running or swimming) to clockwise (tumbling). When chemoattractants bind to the receptors, autophosphorylation of the associated CheA kinases is blocked, reducing CheY phosphorylation, and thereby promoting smooth swimming. Phosphatase CheZ rapidly dephosphorylates CheY. In addition to rapid responses to ligand changes are slower adaptation to baseline chemoattractant levels. This adaptation occurs as methyltransferase CheR and methylesterase CheB methylate and demethylate, respectively, four glutamate residues of the chemoreceptors. The more methylation of a given chemoreceptor, the more active the associated CheA kinase becomes, allowing the system to reset what it considers baseline chemoattractant levels. An additional negative feedback is provided by CheA-dependent CheB phosphorylation, which increases CheB activity.

associates with a number of different chemoattractant-specific chemoreceptors via the adaptor protein CheW. Counteracting CheA is the phosphatase CheZ. In the absence of signals, CheZ maintains CheY in the unphosphorylated state, resulting in the flagella rotating in the counterclockwise direction a majority of the time. However, CheA is active under these conditions, so CheY will occasionally be phosphorylated briefly, resulting in occasional short-lived moments of clockwise rotation. At the cellular level, this behavior manifests as periods of smooth swimming that are roughly one second long interrupted by brief tumbling and direction changes. When a signal binds to one of the many receptors, activity of the associated CheA is suppressed, reducing the frequency with which CheY can be spontaneously phosphorylated, and effectively increasing the length of the periods of counterclockwise rotation of flagella and smooth swimming.

We mentioned earlier that in order for a chemotaxis system to bias movement toward more favorable conditions, the sensing mechanism not only needs to detect chemical signals, but it must also be able to compare its current environment to its previous environment. This memory is achieved through an additional layer of regulation that allows the system to gradually adapt to new baseline concentrations of each chemoattractant being sensed. In each chemoreceptor, there are four glutamate residues that can be methylated by the methyltransferase CheR. The more methylation of a given receptor, the more active the associated CheA kinase. Counteracting this methylation is the methylesterase CheB, which removes the methyl groups. Adaptation of the system arises from the preference of CheR and CheB for a given type of receptor based on the activity of the receptor. That is, CheR preferentially methylates inactive, “kinase off” receptors, while CheB preferentially demethylates active, "kinase on" receptors. As a result, unstimulated receptors will have their kinase activity toned down, while stimulated receptors will require even more chemoattractants to reach the same level of CheA inactivation. Effectively, this CheR/CheB-mediated adaptation resets the baseline level of attractant needed for the system to respond. This adaptation mechanism enables chemotaxis to turn off in the presence of a constant concentration of stimulus and to continuously reset the receptors over time.

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Questions

1. Describe the means by which bacteria sense and respond to changes in their environment.
2. Why would having regulated virulence genes be an advantage to a bacterium?
3. How do simple activators differ from two-component regulatory systems?
4. What type of mutation(s) would cause genes regulated by a two-component regulatory system to be expressed constitutively?
5. How do small RNAs regulate virulence genes?
6. Briefly describe the mechanism by which Rho-independent termination occurs.
7. Compare the autoinducers of Gram-negative and Gram-positive bacteria.
8. Describe how the regulatory mechanism of "quorum sensing" might play a role in the pathogenesis of a *Vibrio* infection and how it works using the example of Lux (bacterial luminescence).

9. Describe how the regulatory mechanism of "quorum sensing" might play a role in the pathogenesis of staphylococcal infection and how it works using the example of the *AgrABCD* locus.

10. Bacteria adapt to changes in their environment and alter regulation of gene expression accordingly. One of the mechanisms of regulation in bacteria involves transduction of an environmental signal. Explain the function of bacterial sensors and transducers for gene regulation. How do two-component regulatory systems control gene expression in response to environmental changes? Give examples.

11. The *Bordetella pertussis* Bvg system is essential for virulence.
    a. What type of system is this, and what are the representative proteins?
    b. How is the Bvg system unique, and how does it become activated?
    c. List two virulence factors that Bvg controls (either directly or indirectly).

12. You are using a gene fusion between a very bad gene (*vbg*) and *lacZ* to study the regulation of *vbg* by iron. (When *lacZ* is expressed, cells are blue; when *lacZ* is not expressed, cells are white.) You introduce a transposon into the strain carrying a *vbg-lacZ* fusion and screen for regulatory mutants. You conclude that *vbg* is expressed in the presence of iron because you observe that, for the wild-type fusions, colonies are ______ in the presence of iron and ______ in the absence of iron. You conclude that the transposon has inserted into a gene encoding an activator protein because you observe that colonies are ______ in the presence of iron and ______ in the absence of iron.
    a. blue … white … blue …blue
    b. white … blue … blue … blue
    c. blue … white … white … white
    d. white … blue … white … white
    e. white … blue … white … blue

13. You are using a gene fusion between a virulence gene (*vir*) and *lacZ* to study the regulation of *vir* by an acidic pH. (When *lacZ* is expressed, cells are blue; when *lacZ* is not expressed, cells are white.) You introduce a transposon into the strain with the *vir-lacZ* fusion and screen for regulatory mutants. You conclude that *vir* is not expressed at an acidic pH because you observe that, for the wild-type fusions, colonies are ______ on an acidic medium (pH 5.0) and ______ on a neutral medium (pH 7.0). In a mutant strain, you conclude that the transposon has inserted into a gene encoding a repressor protein because you observe that colonies are ______ on the acidic medium and ______ on the neutral medium.
    a. blue … white … blue …blue
    b. white … blue … blue … blue
    c. blue … white … white … white
    d. white … blue … white … white
    e. white … blue … white … blue

Solving Problems in Bacterial Pathogenesis

1. You have purified a protein from a bacterium Y that causes cells in tissue culture to increase their cAMP levels. You subsequently cloned a fragment of DNA from bacterium Y that contained two open reading frames, one of which you identified as the gene encoding the protein you isolated, which you called *yctA* (for Y bacterium cytotoxin A). You then determined that expression of the YctA protein occurs only under iron restrictive conditions (i.e., when iron is absent from the medium).
    a. Describe how you would determine if the expression of YctA is regulated by iron at the transcriptional level.
    b. Describe how you would determine whether the other open reading frame, which you have named *yctR*, encodes a possible transcriptional regulator.
    c. If YctR is a transcriptional regulator, how could you determine if it is an activator or repressor protein?

2. The PhoPQ two-component regulatory system of the intracellular pathogen *Salmonella typhimurium*, encoded by the genes *phoPQ*, is one of the most well-studied global regulatory systems and has been shown to regulate the majority of virulence genes involved in intracellular survival in macrophages, resistance to antimicrobial peptides, acid tolerance, and phagosome remodeling to prevent phagolysosome fusion. In *S. typhimurium*, a large number of virulence genes are regulated by a two-component regulatory system, which senses divalent cations, such as Mg^{2+}, Mn^{2+}, and Ca^{2+}, as well as low pH environments. PhoQ is a dimeric transmembrane protein that binds divalent cations to form bridges with membranes that maintain a PhoQ-repressed state. Antimicrobial peptides and acidic pH activate PhoQ. PhoP has an aspartate that when phosphorylated binds to DNA, in particular HilA, the master regulator of *Salmonella* pathogenicity island 1 (SPI-1), and controls expression of

phoP-activated (*pag*) and *phoP*-repressed (*prg*) genes, of which there are over 200.

a. Which of the two components is the sensor kinase and which is the response regulator?
b. What is the phenotype (virulence and expression of regulated proteins) that would result from a null mutation in the response regulator gene?
c. What is the phenotype (virulence and expression of regulated proteins) that would result from a response regulator mutation that led to constitutive activation of the response regulator?
d. Predict what the effects would be for each of the following mutations on activity (ON or OFF) of a *lacZ* fusion with the given gene under the given conditions. *Be sure to state your rationale:*

Effect of transposon insertion into *phoP* on *prg-lacZ* when *Salmonella* is in a phagosome.

Effect of transposon insertion into *phoQ* and constitutive expression of *phoP* from a plasmid on *pag-lacZ* when *Salmonella* is in a phagosome.

Effect of transposon insertion into *phoQ* on *prg-lacZ* when *Salmonella* is in the lumen of the gut.

Effect of transposon insertion into *hilA* on *prg-lacZ* when *Salmonella* is in the lumen of the gut.

Effect of transposon insertion into *phoQ* and constitutive expression of *phoP* from a plasmid on *pag-lacZ* when *Salmonella* is in a phagosome.

3. *Corynebacterium diphtheriae* was the first bacterium for which the symptoms of disease could be explained entirely by the action of a single, secreted protein, diphtheria toxin (DT). DT is produced when iron levels are low. Describe *in detail* how you would determine if the *dtxR* gene encodes a transcriptional activator or repressor of DT gene *(tox)* expression under low iron conditions.

4. A group of researchers have isolated a bacterium Q from the lungs of several cystic fibrosis patients that produces an unusual polysaccharide capsule called QPS. They can see QPS on the surface of the bacteria in electron micrographs from fresh isolates (bacteria just obtained from patients). However, after culturing the bacteria *in vitro* on agar plates, they find that most of the bacteria no longer have QPS on their surface. When the researchers grew the bacteria overnight in culture media, surprisingly they found that some of the bacteria now had QPS on their surfaces. If they then incubated the cultures for longer periods of time, they found that more and more of the bacteria produced QPS on their surfaces. They also found that if the media from old cultures is filtered and added to bacteria lacking QPS on their surfaces, within a short time those bacteria also begin to express QPS on their surfaces.

a. What possible mechanism could account for these observations?
b. The same researchers subsequently cloned two separate DNA sequences, one containing two open reading frames (ORFs), which they named *qpsAB*, and a second containing four ORFs, which they named *qpsCDEF*. The researchers suspected that the first operon may encode two biosynthetic genes responsible for making QPS and the second operon may encode the regulatory system controlling expression of QPS. They then proceeded to conduct a series of experiments to test their hypothesis. The results follow:

   - Introduction of the two *qps* operons into the Gram-positive bacterium, *Bacillus subtilis*, was able to cause expression of QPS on the surface of the bacterium. Deletion of one or more of the *qps* genes resulted in complete loss of QPS expression, even though each of the remaining genes was expressed.
   - Cellular fractionation, followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis using antibodies against the proteins encoded by *qpsC, qpsD, qpsF,* and *qpsE,* showed that QpsD and QpsF were membrane-associated, QpsC was located in the cytosol, and QpsE was found mostly in the medium.
   - The size of the QpsE peptide in the medium (10 residues) was much smaller than its size in the cytosol (45 residues). A synthetic peptide corresponding to the 10-residue peptide could not substitute for a native QpsE peptide.
   - Deletion of QpsF resulted in QpsE being localized as a 45-residue peptide only to the cytosol and not in the medium.
   - Incubation of bacterial membranes with filtered media from old cultures in the presence of [γ-32P]-ATP, followed by SDS-PAGE and autoradiography, revealed that both QpsC and QpsD were radiolabeled. QpsD could radiolabel itself, but QpsC could not.
   - Incubation of cell-free extracts containing QpsC, QpsD, QpsE, and QpsF with oligonucleotides encoding the promoter region of *qpsAB* showed that only QpsC was able to bind the DNA.
   - A series of QpsD-PhoA fusions at various points in the 350-residue QpsD protein were constructed and tested in *B. subtilis*. The results are summarized in Table 1 below:

Table 1. Results of QpsD-PhoA fusions in *B. subtilis*

QPSD-PHOA PROTEINS: POSITION OF FUSION	ALKALINE PHOSPHATASE ACTIVITY	CELLULAR LOCALIZATION OF THE FUSION PROTEIN
Residue 33	+	Medium
Residue 60	–	Membrane
Residue 105	+	Membrane
Residue 142	–	Membrane
Residue 160	+	Membrane
Residue 176	+	Membrane
Residue 200	–	Membrane
Residue 256	–	Membrane

Using the above information, provide a detailed model for QPS regulation in bacterium Q that accounts for all of the observed results. You may draw a diagram if that helps, but be sure to provide a clear role for each of the Qps proteins in your model. Based on the above information, predict the membrane topology of the QpsD protein. Draw a diagram of your model.

5. You are a physician scientist studying the mechanisms by which *Neisseria gonorrhoeae* (Ng) can survive in the human host and are performing a variety of different experiments, including monitoring recurring Ng infections in sexually active males. One of your patients is a sexually promiscuous 22-year-old man who has had gonorrhea five times in the last two years. Each time he visits your clinic to donate bacterial samples during an episode, he is given antibiotics afterward and the infection clears up. Through the course of the study, you have isolated a number of different clinical strains of Ng from this individual.
    a. List two reasons why Ng is capable of causing recurrent infections in this same individual.
    b. Could this patient be vaccinated to avoid these recurring infections? Provide your rationale.
    c. Immunoblot analysis using a monoclonal antibody against the surface protein OpaC shows that OpaC is not detected in Ng strain #1. But you can PCR amplify the *opaC* gene using chromosomal DNA from this strain. Based on sequence analysis of the *opaC* gene, you detect differences at the 5′-end of the gene from this strain compared to a related strain that does produce OpaC. What is the specific type of variation that is occurring in regard to OpaC in this strain? Based on the experimental evidence, what is causing OpaC to not be produced in this strain? Explain *very briefly* how this is most likely occurring.
    d. In Ng strains #2 and #3, you have sequenced the pilin gene *pilE* and have denoted the minicassette variable regions in the gene for strain #2 as A-B-C-D-E-F and for strain #3 as 1-2-3-4-5-6. You isolate chromosomal DNA from strain #2 and add it to a culture of strain #3. After a period of time you isolate colonies from the culture. Sequencing the *pilE* gene from one of the colonies (denoted as Ng strain #4) reveals that you have created a new PilE protein encoded by a gene with the minicassette regions 1-B-C-4-5-6. What has occurred in strain #4 to change the *pilE* minicassette regions to 1-B-C-4-5-6? Will polyclonal antibodies that recognize PilE from strain #2 also recognize PilE from strain #4? Explain.
    e. Another strain (denoted as Ng strain #5) isolated from your patient's most recent visit appears to be a new strain that does not have pili (Pil⁻ phenotype). Using polyclonal antibodies generated against PilE proteins from strains #2 and #3, you are able to detect a PilE protein in cell extracts from strain #5 that is much larger than the PilE from strains #2 or #3, but this pilin is not detected on the surface of the bacteria. What event has occurred in strain #5 to give you the Pil⁻ phenotype? Explain why there is a Pil⁻ phenotype with strain #5 even though you can detect the presence of PilE protein.
    f. On the last visit from your patient, you also collect some blood and find that there are both IgM and IgG antibodies present in the patient's serum. You further find that the IgG antibodies recognize PilE from all of the strains, whereas the IgM antibodies do not recognize any of the PilE proteins. Interpret these results.

6. You are a senior research scientist working for a large agricultural firm that is interested in novel, nonpesticide ways of controlling plant insects. Your research group is studying pea aphids (plant insects) that possess several types of symbiotic bacteria. Pea aphids often harbor as a symbiont *Hamiltonella defensa*, which is a Gram-negative bacterium related to *Salmonella*. It is believed that colonization with *H. defensa* confers substantial resistance to parasitic wasps, which are natural enemies of the aphids that lay their larva inside the aphids. In aphids harboring *H. defensa*, the wasp larvae die prematurely, allowing the aphid host to develop into adults and reproduce. Recent genome sequencing data have revealed that *H. defensa* possesses two pathogenicity islands (PAIs) that are prophages, each encoding a type III secretion system (T3SS) and putative virulence genes. The first PAI, called APSE-1, encodes a homolog of the catalytic subunit of Shiga toxin (*stx*) produced by *Shigella*, which inhibits protein synthesis by disrupting 28S rRNA. The second PAI, called APSE-2, encodes a homolog of the catalytic subunit of the cytolethal distending toxin (*cdtB*) produced by *Campylobacter jejuni*, which is a DNase with a nuclear localization sequence that targets it for the eukaryotic nucleus, where it interrupts cell cycle progression by arresting the cells in G2 phase. It is possible that these APSE-encoded toxins might be selectively targeted to destroy larval cells

of attacking parasitic wasps. However, neither of these PAIs encodes genes that might be the corresponding binding subunits of the two putative toxins, suggesting that the binding subunits are novel and not recognizable by sequence comparison or that the Stx- and CdtB-like proteins might instead be delivered to the wasp cells by the T3SS. To further characterize this fascinating symbiotic relationship, you have developed a system for genetically manipulating *H. defensa* that is based on *Salmonella* genetic tools, as well as a system for growing wasp larva cells in tissue culture.

a. Describe how you would experimentally go about demonstrating that the Stx-like and CdtB-like proteins are indeed delivered by the *H. defensa* T3SS into the wasp cells and that they end up in the expected cellular locations. Be sure to include experimental design, reagents used, and how you visualized and interpreted the data. (Hint: You may need more than one type of experiment to demonstrate this.)
b. It is thought that binding of the *H. defensa* to a surface antigen on the wasp larva cell triggers the expression of the CdtB gene. Describe in detail how you would confirm quantitatively that CdtB expression is regulated at the transcriptional level by the wasp larva cell surface antigen.
c. Describe how you would go about identifying potential regulatory proteins of *cdtB* expression. Be sure to provide the reagents, screening conditions, how you would visualize/measure the results, and how you would verify the results.
d. Using your strategy, you identify three genes (which you name *cdtR, cdtS*, and *cdtT*) that might be regulators of *cdtB* expression. Describe in detail how you would determine if the gene product of *cdtR* is a transcriptional activator or repressor of *cdtB* gene expression.
e. To demonstrate that the gene products of *cdtR* and *cdtS* directly regulate the *cdtB* gene, you purify the corresponding CdtR and CdtS recombinant proteins in *E. coli* and perform an experiment to demonstrate that they can bind to the promoter region of the *cdtB* gene. However, your results show that only CdtR can bind to the DNA. Provide (with rationale) a function for CdtS and at least one additional experiment that could be performed to confirm your hypothesis.
f. Despite numerous attempts, you discover that you cannot visualize the predicted CdtT protein in Western blot analyses of bacterial cells, yet you were able to observe transcriptional regulation of the *cdtB* gene using your reporter system that you made above. After further analysis of the *cdtT* gene sequence, you discover that part of the sequence is complementary to the 5′ promoter regions of a *H. defensa* polysaccharide biosynthetic gene and several adhesin-like genes. Provide (with rationale) an explanation for these observations.

7. *Clostridium perfringens* is a Gram-positive anaerobic pathogen that causes serious human and animal diseases, including clostridial myonecrosis (i.e., “flesh eating” disease) and gas gangrene (i.e., “tissue rotting”). *C. perfringens* produces many extracellular enzymes and toxins, including alpha-, theta-, and kappa-toxins, that are encoded by the genes, *plc*, *pfoA*, and *colA*, respectively. Being interested in understanding the regulation of these toxin genes, you performed STM and isolated a number of transposon-insertion mutants that are avirulent in a mouse model of gas gangrene infection. Sequence analysis of these mutants revealed that five of these avirulent mutants, Cpmut1-Cpmut5, were in genes unrelated to the toxin genes, yet these mutants no longer produced the three toxins. Four of the five putative regulatory genes (*mut2-mut5*) were found in a single operon. You then deleted each of these genes and designated the resulting mutants as CpΔmut1 through CpΔmut5. You also cloned each of the genes and found that you could express the corresponding proteins in *E. coli* as well as *C. perfringens*. You were interested in characterizing these mutants and determining the functional identity of the proteins encoded by the putative regulatory genes, so you designed a series of experiments.

a. In the first experiment, you determined the cellular localization of the proteins encoded by the genes *mut1* through *mut5*. Describe how you conducted this experiment. Be sure to include experimental design, reagents used, and how you visualized and interpreted the data.
b. In the second experiment, you found that addition of cell-free filtrates from overnight cultures of wild-type *C. perfringens* or any of the mutants CpΔmut2-CpΔmut5 to freshly inoculated cultures of the Gram-negative bacterium *Vibrio harveyi* greatly stimulated the luminescence of the *V. harveyi* cells, whereas overnight culture filtrates of CpΔmut1 had no effect on *V. harveyi* luminescence. You found that overnight culture filtrates from *E. coli* expressing *mut1*, but not *mut2-mut5*, could also stimulate *V. harveyi* luminescence. Provide a possible explanation for these results. What is the most likely identity of the *mut1* gene? Be sure to state your rationale. Provide at least one additional experiment that you might perform to confirm your conclusion.
c. In the third experiment, you find that although CpΔmut2, CpΔmut3, CpΔmut4, and CpΔmut5 are all defective in making the three toxins (alpha-, theta-, and kappa-toxins), when you add overnight culture filtrates of wild-type *C. perfringens*, CpΔmut2, or

CpΔmut5 to cultures of CpΔmut3 or CpΔmut4, all three toxins are produced. However, when overnight culture filtrates of CpΔmut3 or CpΔmut4 are added to cultures of CpΔmut2 or CpΔmut5, no toxin is produced. Cellular fractionation results indicate that both Mut2 and Mut3 are cytoplasmic proteins, while Mut4 and Mut5 are integral membrane proteins. Provide a possible explanation for these results. What is the most likely identity of each of the *mut2-mut5* genes? Provide at least one additional experiment for each of the proteins Mut2-Mut5 that you might perform to confirm your conclusions. (Hint: You should describe at least four separate experiments, one for each of the *mut*-encoded proteins.)

8. You are a graduate student who has been performing a transposon mutagenesis screen on the medically important bacterium, *Americana badbugium*. Based on previous experimental evidence, this pathogen encodes a virulence-associated type III secretion apparatus that is turned on when *A. badbugium* is growing at 37°C and turned off when *A. badbugium* is growing at 23°C. Although a cytotoxic effector protein (CepA) of this secretory machinery has been defined, the other proteins required for secretion have not been identified. You have found a transposon mutation that results in an attenuated phenotype *in vivo* and are interested in characterizing this mutant for your doctoral dissertation. You develop a series of experiments to characterize the protein and obtain various results for the mutants. Use the above information and the results listed under each mutant to answer questions for that particular mutation. The results from these studies are:

   - In the wild-type bacterium, CepA is expressed and secreted when the bacterial cells are grown at 37°C and upon bacterial contact with host cells.
   - In the mutant bacterium, CepA is not expressed and not secreted.
   - You have determined that all structural components of the type III secretion system are intact and functioning properly.
   - You determine that *cepA* mRNA is not detected when the mutant bacteria are growing at 37°C.
   - Very low levels of *cepA* mRNA are detected when the mutant bacteria are growing at 23°C.
   - You have identified and sequenced the gene that is mutated and have expressed the recombinant protein. You find that the N-terminal domain features several regions of conserved amino acids that are similar to other proteins that function in a similar manner.
   - Using transcriptional fusions, you have demonstrated that the recombinant protein, encoded by your gene of interest, can bind to the *cepA* promoter and is required for transcriptional activation of *cepA* when the bacterial cells are growing at 37°C.
   - The defect in *cepA* transcriptional activation and secretion at 37°C is complemented by expression of your gene of interest (on the chromosome or on a plasmid) in the mutant bacterium.

   a. What type of protein have you identified?
   b. Propose a function for your gene/protein of interest.
   c. When is your protein/gene activated in *A. badbugium*?
   d. What other protein is required for your protein to function properly, and how does this additional protein function?

9. Chemotactic responses in *E. coli* and *Salmonella* are regulated through the Che signal transduction pathway. Swimming behavior can be affected by mutations in the pathway. For each of the following mutations, indicate the phenotype you would expect the mutant to exhibit. Choose from phenotypes A through C, using each phenotype as many times as necessary.

   A. runs (swims smoothly) constantly; never changes direction (i.e., never tumbles under any conditions)
   B. tumbles constantly under normal conditions but can run in response to high concentrations of stimulant
   C. runs constantly under normal conditions but can tumble in response to high concentrations of repellent

SITE OF MUTATION	PHENOTYPE
a. *cheZ*, the phosphatase gene; mutation results in loss of phosphatase activity	
b. *cheY*, the response regulator gene; mutation results in loss of the conserved aspartate (Asp, D)	
c. *cheR*, the methyltransferase gene; mutation results in loss of methyltransferase activity	
d. *cheA*, the histidine protein kinase (sensor) gene; mutation results in loss of the conserved histidine (His, H)	
e. *cheB*, the methylesterase response regulator gene; mutation results in loss of the conserved aspartate (Asp, D)	

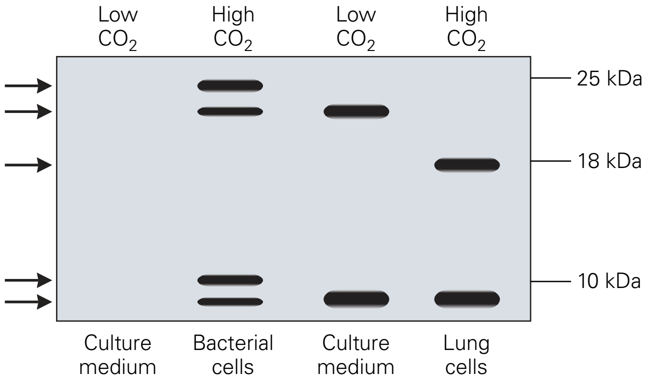
Special Global Perspective Problems: Integrating Concepts in Pathogenesis

1. A group of researchers at the university veterinary diagnostics laboratory isolated a new bacterium from the blood and stool of several horses that became ill and died at a local farm. Prior to death, symptoms included disorientation, loss of motor function, and flaccid paralysis, so the researchers suspected central nervous system (CNS) involvement and possible production of neurotoxin. Based on 16S rRNA comparison, they found that the new bacterium is related to the Gram-positive bacterium *Clostridium botulinum,* and they subsequently named it *Clostridium equiniae*. They also determined that the bacterium is sensitive to metronidazole, but resistant to beta-lactam antibiotics such as penicillin or to macrolide antibiotics such as erythromycin and azithromycin. However, although *C. equiniae* is resistant to azithromycin, the researchers found that when they treated infected mice with metronidazole in combination with azithromycin, the LD_{50} value went from 10 for control without antibiotic treatment to 10^4 for treatment with metronidazole alone to 10^7 for combined treatment of metronidazole plus azithromycin, and the mice recovered from paralysis much sooner with the combined treatment than with metronidazole treatment alone. Interestingly, when they were plating out the bacteria to determine the LD_{50} values, the researchers observed that the colonies isolated from the control mice and mice treated with metronidazole alone were mucoid, but those from mice treated with both antibiotics were not.
   a. What possible mechanism(s) could account for all of these observations (i.e., change in LD_{50} value, faster recovery from paralysis, and change in mucoid phenotype)? Be sure to provide your rationale.
   b. Provide an experiment that could be performed to confirm your hypothesis.

2. A group of researchers are studying a Gram-negative respiratory pathogen, called bacterium K. During the course of their studies they identify a secreted protein cytotoxin (which they call Kct). They determine that Kct causes increased phosphorylation of MAPK proteins and cellular proliferation in cultured lung cells. They generate rabbit polyclonal antisera against filtered, dialyzed, and concentrated culture filtrates from bacterium K and determine that these antibodies neutralize the toxin and protect animals from disease. They separate the proteins present in bacterial culture filtrates by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transfer the proteins to a membrane, and detect the proteins by performing Western blot analysis using the rabbit antisera against Kct. The Western blot analyses reveal that the antibodies are reactive against two proteins produced by the bacterium—one with a size of 80 kDa and another with a size of 20 kDa. Both of these proteins are secreted into the medium only when they are grown under *in vivo* conditions, including temperature (37°C) and high blood/tissue levels of CO_2. Using an *IVET* screening strategy, they subsequently clone three fragments of DNA from bacterium K that each contains an operon important for production and secretion of the toxin, and hence virulence.
   - The first operon has two ORFs: one (which they call *kctS*) has five membrane-spanning hydrophobic sequences in its N-terminal domain and some sequence homology to protein kinases in its C-terminal domain; the other (which they call *kctR*) has no significant sequence homology to any protein in the database, but it does have a helix-loop-helix secondary structure motif that is frequently found in DNA-binding proteins.
   - The second operon has two ORFs, which they call *kctBA*. They suspect these genes encode the protein toxin they isolated, since the first ORF encodes a protein of 85 kDa that has some sequence homology to the B subunit of anthrax toxin and the second ORF encodes a protein of 25 kDa that has some homology to mammalian protein kinases.
   - The third operon has 13 ORFs, which they name *kctC* through *kctO*. Many of the proteins encoded by these genes have membrane-spanning hydrophobic sequences indicative of integral membrane proteins; some also have sequence similarity to proteins involved in biogenesis of type IV pili, and one has an ATP-binding motif.

   a. The researchers determine that transposon insertion into *kctS* removes the ability of CO_2 to regulate expression of Kct. Describe (in detail) how the researchers could determine if the expression of Kct under physiological CO_2 conditions is regulated at the transcriptional level by a two-component regulatory system consisting of KctS and KctR, and how they could determine if *kctR* encodes a transcriptional activator or repressor of Kct gene expression under physiological CO_2 conditions. Based on the given information, what is the predicted result?
   b. The researchers perform cellular fractionation studies of toxin production in the presence of lung cells and examine the protein content of the fractions by using SDS-PAGE and Western blot analyses. Provide a detailed explanation (with rationale) and interpretation for the results shown in the Western blot. Be sure to also provide an explanation for the difference between the predicted size of the A and B subunits of Kct and the

size of the proteins found in the bacterial culture medium and associated with the mammalian lung cells (i.e., how does this occur?). What evidence could they obtain from sequence analysis that would support your explanation?



c. Further studies reveal that Kct action on lung cells requires a low-pH-dependent step, since agents that prevent acidification, such as basic buffers, block toxin effects. Based on the given information above and these results, propose a molecular mechanism by which Kct might cause the observed increased mitogenesis in lung cells. Provide details of each of the steps involved.

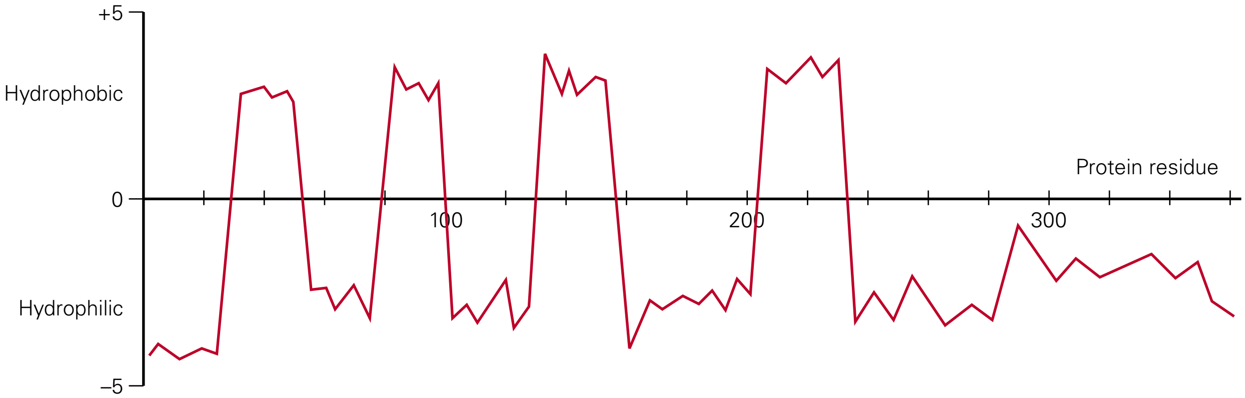
d. To determine the role of the third operon (*kctC-O*) in Kct production, the researchers make in-frame deletions of each of the genes. They then perform fractionation studies of toxin production in the presence of lung cells at high CO_2, followed by SDS-PAGE and Western blot analyses, and find that in each case the Kct A and B subunits are not secreted into the medium, but instead localize to the periplasm with molecular sizes of 20 kDa and 80 kDa, respectively. Provide a detailed explanation for these results.

3. *Bacillus anthracis*, the causative agent of anthrax disease, is a spore-forming Gram-positive bacterium that is touted as a top choice for a biological weapon because of its lethality (anthrax is almost always deadly if not treated early) and because the spores are colorless, tasteless, odorless, extremely hardy (can be stored dry for decades without losing viability), and can be easily dispersed (even through the mail). There are three forms of the disease: cutaneous anthrax acquired through cuts or abrasions of the skin that is characterized by swelling (edema), intense but painless inflammation, and a necrotic (dying) center that turns black; intestinal anthrax (usually caused by consumption of contaminated meat), which is characterized by an acute inflammation of the intestinal tract, abdominal pain, vomiting of blood, and severe diarrhea; and systemic anthrax (or inhalational anthrax), which is characterized by generalized edema and massive pulmonary edema and hypotension (low blood pressure) and is nearly always fatal once symptoms occur with the victim usually succumbing to toxic shock, heart failure, and/or encephalitis. Epidemic outbursts of inhalational anthrax are not natural and so can only be attributed to the deliberate release of a massive penetration of spores into the atmosphere by humans. The major virulence factors of anthrax are: the A3B7 anthrax toxin complex of the A subunits lethal factor (LF, a zinc-dependent metalloprotease) and edema factor (EF, a calcium-calmodulin-dependent adenylate cyclase) and the B subunit protective antigen (PA) that is encoded on a large 184-kb virulence plasmid called pXO1 and an anti-phagocytic poly-γ-D-glutamic acid capsule for which biosynthetic genes are encoded on a large 97-kb virulence plasmid called pXO2. While the three toxin genes for LF (*lef*), EF (*cya*), and PA (*pagA*) are located on the same plasmid, they are not part of the same operon and appear to have their own promoters. The capsule genes (*capBCADE)* are all part of the same operon.

a. The anthrax toxin subunits are secreted into the medium by the general secretory pathway. What feature of the sequences of these proteins would support this conclusion? Describe how you might demonstrate experimentally that they are indeed secreted by the general secretory pathway. Be sure to provide an example of what the expected resulting data might look like and your rationale.

b. Describe how you might demonstrate experimentally that anthrax toxin is indeed an AB toxin, where PA is the B subunit that delivers both LF and EF to the cytosol. Show your expected results.

c. *In vivo*, the signals that stimulate expression of anthrax virulence genes, the *lef, cya*, and *pagA,* are thought to be temperature (ON at 37°C) and blood/tissue levels of carbon dioxide (ON at 5% CO_2). Transposon insertion into *atxA* removes the ability of CO_2 to stimulate expression of anthrax virulence genes. Describe how you would determine if the expression of the *lef, cya*, and *pagA* genes under physiological conditions is regulated at the transcriptional level and if AtxA is a transcriptional activator or repressor of anthrax virulence gene expression under high CO_2 conditions. Be sure to provide the reagents and conditions used, how the results will be visualized or measured, and what the expected results would be for these genes. Based on the given information, what is the predicted result?



d. You suspect that there is a global regulator of virulence gene expression that senses physiological conditions. Describe how you would go about finding this putative regulator of both toxin production and capsule biosynthesis. Be sure to provide the reagents and conditions used in your screen, how the results from your screen will be visualized or measured, what the expected results would be, and how you would distinguish between regulators of the toxin genes alone, the capsule biosynthetic genes alone, and regulators of both (i.e., the putative "global" regulator).

e. From your screen, you identify seven putative regulatory genes (denoted as *bamut1-bamut7*). The *bamut1* gene is immediately downstream of *pagA*, and these two genes appear to be part of the same operon. You suspect that the gene product of *bamut1* might be a regulator of *pagA,* so you rename it *pagR*. Describe how you would determine if the gene product of *pagR* is an activator or repressor of *pagA* and whether it acts as a protein or as an sRNA.

f. Deletion of *pagR* results in increased basal expression of *pagA* at low temperatures and low CO_2 levels, but under physiological conditions, *pagA* expression is just as high as wild-type without deletion of *pagR*. Provide an interpretation with rationale for these results.

g. The *bamut2* deletion mutant is no longer stimulated by CO_2 to express *lef, cya, pag*, or *capBCADE*, whereas in the *bamut3* deletion mutant, the expression of these genes is constitutively ON. Provide an interpretation with rationale for these results.

h. Toxin production is also controlled by growth phase (i.e., toxin production is highest during exponential phase, but once the bacteria reach stationary phase toxin production is turned OFF). What mechanism could account for this observation? Provide your rationale.

i. The *bamut4-bamut7* genes are all located on the same operon, and toxin production by the mutants deleted in any one of these genes is no longer regulated by growth phase. Analysis of the protein sequence of these genes reveals that none of them have any significant sequence homology to known proteins in the databases, but there are some sequence motifs. Protein sequence analysis of *bamut4* reveals that it has four membrane-spanning sequences in its N-terminus and an ATP-binding kinase-like motif in its C-terminus. The protein encoded by *bamut5* has a helix-loop-helix secondary structure motif that is found in DNA-binding proteins. The protein encoded by *bamut6* has an ATP-binding motif and six membrane-spanning hydrophobic sequences. The protein encoded by *bamut7* is a small protein of 50 amino acids with a stretch of 23 hydrophobic residues at the N-terminus. Predict the function and cellular location of each of the proteins encoded by *bamut4-bamut7*, and provide one additional experiment for each that could be performed to confirm your prediction for each.

j. Using the information from the hydrophobicity plot, design an experiment that would allow you to determine the membrane topology of the Bamut4 protein. Be as specific and efficient as possible, and provide details of your experimental setup, as well as your expected results. Draw a model of the membrane topology of your protein.

k. Antibiotics will suppress infection only if administered very early after exposure, usually within the first two days. After that, there is no effective treatment for unvaccinated individuals of inhalation anthrax. By the time symptoms manifest, it is highly likely that death will occur despite the best efforts of modern medical science (i.e., the mortality rate is 50% if treated within the first three to five days after symptoms appear, 100% thereafter). Ciprofloxacin (Cipro) is the preferred antibiotic, but penicillin, erythromycin, tetracycline, chloramphenicol, and clindamycin can be used. Recent studies show that patients with inhalation anthrax receiving pleural (lung) fluid-drainage treatment and multidrug antibiotic therapy with a combination of ciprofloxacin, clindamycin, and Augmentin (ampicillin plus clavulanic acid) have significantly higher survival rates. Provide a possible explanation for this observation, including the rationale for the inclusion of each component in the treatment regimen.

15

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CHAPTER 15

Antimicrobial Compounds and Their Targets

Antibiotics are indeed "wonder drugs." It is now hard to imagine the era, prior to World War II, when most bacterial diseases were life-threatening. Back then, bacterial infectious diseases and infections of simple wounds or surgical wounds acquired during operations in hospitals or in childbirth could rapidly spin out of control and result in death. The appearance of antibiotics, first sulfa drugs and then penicillin, heralded a new and exciting era of antibiotic discovery and development that completely revolutionized human and animal health, to the point at which there are relatively few bacterial diseases that we have been unable to cure. Unfortunately, it looks like this era may now be over. What happens if—for the first time in history—we lose a cure? This scenario is already on the horizon for a few bacterial diseases and will likely result in a drastically negative view of public health care systems, regulatory and funding agencies, and the pharmaceutical industry.

Antimicrobial Compounds: The Safety Net of Modern Medicine

The Importance of Antimicrobial Compounds

The innate and adaptive defenses of the body are remarkably effective, but they are not perfect. Not only have bacteria developed ways of circumventing them, but they also have ways of taking advantage of instances in which there is disruption of the defenses, such as during surgery or cancer chemotherapy, when the body is susceptible to infection. Such medical disruptions offer great health benefits if the risk of subsequent infection can be minimized. One of the greatest advances in human health during the past century was the discovery that our natural defenses could be augmented with externally provided chemical defenses: **disinfectants** (applied to nonliving objects or surfaces), **antiseptics** (applied to living tissues or skin), and **antibiotics** (administered outside and inside the body).

The public has taken antimicrobial compounds (antiseptics, disinfectants, and antibiotics) so much for granted that they have forgotten how crucial these compounds are to human well-being. Only with the appearance of antibiotic-resistant bacteria have physicians and public health officials begun to recall how essential the effectiveness of antibiotics is to modern medicine. Without antibiotics, nonessential surgical procedures, such as knee and hip replacements, could not be done on a routine basis. Essential surgical procedures, such as bypass surgery or heart valve replacement, would become a lot riskier for the patient, who would be put in the unenviable position of having to decide whether to risk death because of failure to undergo the operation or to risk death due to an overwhelming infection.

Surgical procedures have been practiced in emergency situations for centuries, but the patients often died from ensuing infections. In the 1800s and early 1900s, surgeons discovered how to reduce postsurgical infections dramatically by using sterilizing procedures, such as vaporized phenol (carbolic acid), in the operating room. A surgeon of that era owed his success not only to his skill at wielding the knife, but also to his ability to endure the effects of repeated phenol exposure. Only when antibiotics and disinfectants made phenol a thing of the past did surgery begin to flourish and medical students begin to vie for admission to the specialty.

Unfortunately, just as virulence factors enable bacteria to evade or undermine the natural defenses of the human body, so the adaptability of bacteria has enabled them to devise strategies for evading these chemical defenses. These strategies, called **mechanisms of antimicrobial resistance**, have the potential to undermine many of the advances we have made over the past century. In this chapter, we will first examine the importance of antiseptics and disinfectants and their mechanisms of action. We will then explore how we go about discovering new antimicrobial compounds and new bacterial targets for potential antibiotic development. We will also cover how the major classes of current antibiotics work and the means we are using to find alternative strategies to combat bacterial infections in the face of antibiotic resistance. How bacteria become resistant to antibiotics is the subject of chapter 16.

Avoiding, Reducing, and Preventing Exposure

We have said it before (see chapter 2), but it still holds: The best and most effective strategy for preventing infections is to prevent the pathogen from entering the body in the first place. Physical sterilization methods still serve as highly effective means of reducing the number of microbes present, thereby reducing the transmission of microbes, and their importance for controlling infectious diseases should not be discounted.

Chlorination. Effective water treatment practices that reduced the incidence of waterborne diseases, such as cholera (caused by *Vibrio cholerae*) and typhoid fever (caused by *Salmonella enterica* serovar Typhi), were not in place until the twentieth century and, indeed, safe drinking water is still lacking in many parts of the world today. Bacterial counting and filtration methods to detect and remove the presence of *Escherichia coli* and other enteric bacteria (**coliforms**) in drinking water were first developed in the late 1800s and implemented in major cities around the turn of the twentieth century. This was followed shortly afterward with the use of chlorination as a disinfectant treatment for large water supplies, especially in cities. For example, in Philadelphia, Pennsylvania, a system for connecting several water facilities through pipelines and sand filtering the city water supplies was introduced in 1906, followed by use of water chlorination in 1913 (Figure 15-1). Each of these measures to decontaminate the city's water supplies dramatically reduced the incidence of typhoid fever to such an extent that, by the 1930s, city health officials concluded that most cases were acquired outside of the city.

Heat Treatment. Heating can inactivate or kill all kinds of microbes, as well as viruses and toxins, depending on the method used and the temperature, pressure, and duration of application. Not all methods employed result in complete sterilization. For example, **pasteurization** is frequently used in the food industry to slow food spoilage due to microbial growth. Commercial methods are standardized and controlled by the Food and Drug Administration (FDA) or the U.S. Department of Agriculture (USDA) in the United States. Foods, particularly milk and dairy products, are pasteurized by heating for a specified length of time at a specified temperature, typically 72°C for 15 to 20 seconds or, for extended shelf life when tolerated, 135°C for one minute, and then cooled. Unlike other sterilization methods, not all of the microbes are killed by pasteurization; their numbers are simply reduced so as to delay spoilage.

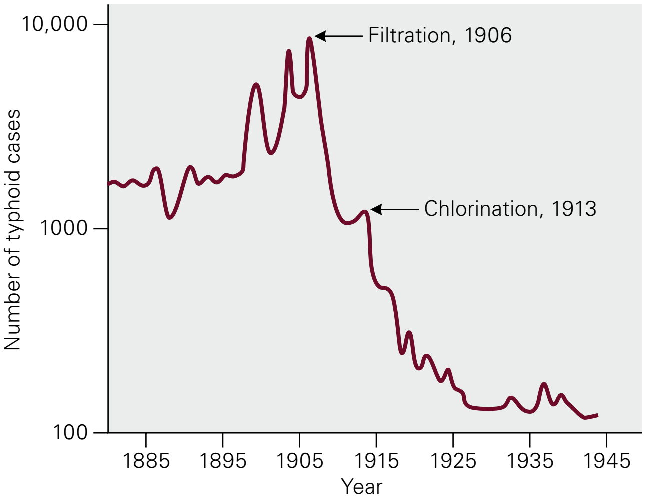


Figure 15-1. The effect of introduction of water treatment practices on the incidence of typhoid fever. With the introduction of water filtration in 1906 and then chlorination in 1913, the annual number of cases of typhoid fever, caused by *Salmonella enterica* serovar Typhi, in Philadelphia, Pennsylvania, was dramatically reduced.

Moist-heat sterilization involves **boiling**, typically at 100°C for more than 10 minutes, which kills most bacteria. Boiling for 30 minutes will kill virtually all vegetative bacterial cells, but will not always kill spores. Indeed, heating triggers many bacterial spores to germinate shortly afterward, converting to vegetative cells that then grow. In many canning practices, a modified method, called **tyndallization**, uses three consecutive steam treatments over the course of three days to achieve sterilization that kills any spores present. Pressure cooking or **autoclaving** uses steam heat under pressure to sterilize. Pressure sterilization, typically at 121°C and 20 psi for 20 to 60 minutes, is the prevailing method used for medical or microbiological laboratory sterilization that requires aseptic techniques. The pressurized heat denatures microbial proteins and nucleic acids and often lyses cells and spores at longer treatment times. However, some infectious proteins (e.g., prions) can survive autoclaving, depending on the conditions.

Baking or dry-heat sterilization usually involves **conduction heating** in an oven, where heat is applied to the exterior surface, typically greater than 150°C for one to two hours, and in the process the entire item is heated to a desired internal temperature that denatures proteins. Dry-heat sterilization is often used for inanimate objects that can withstand high temperatures for long periods of time.

Ionizing Irradiation. Ionizing irradiation, also known as cold pasteurization, can eliminate disease-causing bacteria and parasites in food by treating the food or other items with approved levels of ionizing (high-energy) radiation that damages the microbial DNA. Irradiation is currently used for various foodstuffs (spices, fruit, potatoes, mushrooms, vegetables, poultry, meat), medical devices, cosmetics, feminine hygiene products, infant consumables, pet treats, animal vaccines, and astronaut supplies. Commercialized ionizing irradiation methods are standardized and controlled by the FDA or USDA in the United States (Table 15-1).

There are three types of irradiation technologies currently used. **Gamma (γ) rays** generated from radioactive material, typically cobalt-60 or cesium-137, are used to produce high-energy photons that penetrate the target material. (Note: γ rays do not make the target material radioactive.) Currently, this technology is used to sterilize medical, dental, and household products and has some applications in the food industry. **Electron beams**, a stream of high-energy electrons produced by an electron gun (no radioactive material involved), can penetrate up to about 3 cm and have been used to sterilize medical devices for more than 15 years. This technology is gaining some interest in the food industry. **X rays**, produced by directing an electron beam at a thin plate of gold or other metal, are being developed by the food industry as a sterilization technology because they can penetrate up to 38 cm. The instruments used are usually more powerful than medical X-ray machines.

The dose of irradiation is measured in **grays** (Gy, 1 Gy = 1 joule/kg), the amount of energy transferred to food, microbe, or other substance. The size of the DNA is a factor in how readily a microbe is killed by irradiation. Parasites and insects are rapidly killed, with a dose of 0.1 kGy killing over 90% of the organisms. Bacteria alone are typically killed with doses in the range of 0.3 to 0.7 kGy, but higher doses are required for bacteria embedded in food. For example, the dose needed to kill 10^4 to 10^6 *Salmonella* in a piece of chicken is about 4.5 kGy and in juice is about 3 kGy. The dose needed to kill anthrax spores is about 30 kGy. Viruses also require doses greater than 10 kGy, and proteins (toxins, prions) or other small molecules are not affected at all.

The effects of irradiation on food and the animals or humans that consume the irradiated food have been studied extensively. The conclusion is that when food irradiation is used at appropriate levels the irradiation drastically reduces or eliminates disease-causing microbes. Food irradiation is a safe and effective technology that can prevent most foodborne diseases, whether through natural, accidental, or deliberate introduction. The food itself does not become radioactive or generate toxic substances, and its nutritional value is essentially unchanged, while increasing the shelf life of the irradiated food.

Table 15-1. History of food irradiation technology usage

APPROVAL YEAR	FOOD	DOSE (kGy) PERMITTED	PURPOSE
1963	Wheat flour	0.2–0.5	Control of mold
1964	White potatoes	0.05–0.15	Inhibit sprouting
1986	Pork	0.3–1.0	Kill *Trichinella* parasites
1986	Fruits and vegetables	1.0	Insect (fruit flies) control, increased shelf life
1986	Herbs and spices	30.0	Sterilization
1990—FDA	Poultry	3.0	Bacterial pathogen reduction
1992—USDA	Poultry	1.5–3.0	Bacterial pathogen reduction
1997—FDA	Red meat	4.5	Bacterial pathogen reduction
1999—USDA	Red meat	4.5	Bacterial pathogen reduction
2008—FDA	Lettuce and spinach	4.0	Bacterial pathogen reduction, increased shelf life

Killing versus Inhibiting Growth

The general term “antimicrobial” is commonly used for any substance or drug that can kill or inhibit the growth of microbes, and antimicrobials can include compounds that control bacteria and other microbes or viruses. There are more specific terms for antimicrobials that selectively act on a particular type of microbe: **antibacterials** or **antibiotics** for bacteria; **antifungals** for fungi (molds or yeasts); **antiparasitics** for protists, worms, or other parasites; and **antivirals** for viruses.

A key concept in antimicrobial therapy is the difference between microbicidal and microbiostatic compounds. Antimicrobial compounds that kill the microbe above a certain dose are called **microbicidal**. Others, called **microbiostatic** compounds, merely stop or slow the growth of the microbe, but do not kill it. Similarly, antibacterial compounds that kill bacteria are called **bactericidal**, while **bacteriostatic** compounds only stop or slow the growth of bacteria. With bacteriostatic agents, the bacteria are not killed and can frequently recover once the antibiotic is no longer in use (referred to as **tolerance**). In patients with an intact immune system, bacteriostatic compounds can be very effective because the defenses of the body only need a little help in slowing the growth of bacteria so that they can be eliminated from the body. In people with defective immune defenses, however, there is much greater reliance on the antibacterial compound to effect a cure. Bactericidal antibiotics are often best for treating infections in such patients.

At one time, the distinction between bactericidal and bacteriostatic compounds seemed clear-cut. Certain antibacterials were bacteriostatic, others were bactericidal. That is, the distinction resided in the properties of the antibacterial compound. It is now clear that properties of the bacteria can affect this distinction too. A good example of this is bacterial biofilms. Bacteria that form biofilms change metabolically into a less active state. Such bacteria are able to resist (i.e., tolerate) an antibiotic that would be bactericidal if the bacteria were dividing rapidly. In other words, a bactericidal antibiotic is rendered bacteriostatic or completely ineffective by the growth state of the bacteria. This is the reason biofilms that form on medical implants and catheters are so difficult to eliminate.

Tests Used To Assess Antibiotics

The susceptibility of bacteria to antibiotics and other antibacterial compounds is measured by simple growth tests. In one version, bacteria from freshly grown cultures are diluted into a row of wells of a microtiter plate containing rich growth medium. Different dilutions of the compound to be tested are then added to the wells and the bacteria are allowed to grow overnight. Bacteria in the control well that lacks the compound and in wells containing low concentrations of the compound will grow to a stationary phase, making the well appear turbid. Above a certain concentration of the compound, the bacteria will fail to grow and the well will appear clear. This concentration of the compound is defined as the **minimal inhibitory concentration (MIC)**. The lower the MIC, the more potent the compound is as an inhibitor. For a bacteriostatic compound, the bacteria in the clear wells can be recovered because their growth has merely been inhibited. At low concentrations, even bactericidal compounds may inhibit growth without completely killing the bacteria, but at higher concentrations all of the bacteria will be killed. This concentration is referred to as the **minimal bactericidal concentration (MBC)**. Thus, the MBC is greater than or equal to the MIC for bactericidal compounds.

This simple dilution susceptibility assay format to determine MICs can be performed simultaneously on different species of bacterial pathogens growing in the same microtiter plate. These comparisons show that different bacterial pathogens are susceptible to a range of concentrations of a given antibiotic. For the most susceptible bacteria, the MIC may be less than 0.01 μg of antibiotic per mL of culture. Some antibiotics, such as vancomycin, will only inhibit Gram-positive, but not Gram-negative, bacteria because these compounds fail to penetrate the outer membrane of Gram-negative bacteria. For these compounds, all of the wells will be turbid in the Gram-negative bacteria MIC assays. Importantly, MIC values provide a measure of resistance, because resistance to an antibiotic leads to an increase in the MIC value for a given bacterial species. The MIC assay can also be used to test whether compounds of interest bind to carrier proteins in blood, such as serum albumin. Tight binding to carrier proteins can be a serious hurdle for a compound intended to act as an effective antibiotic because the compound may not be free to attack bacteria in mammalian hosts. When a compound binds tightly to serum albumin, its MIC will increase in assays containing the protein compared to those lacking the protein.

Besides the broth format, there are other formats of antibiotic susceptibility assays, such as the **Kirby-Bauer disc diffusion assay**. Here, the bacteria are spread on agar plates, and wafer discs or strips are placed on the agar with different concentrations of the antibiotic being tested, followed by overnight incubation to observe bacterial growth on the plate (Figure 15-2). Susceptibility is based on the zones of growth inhibition that are present around the discs or strips containing the antibiotic, where more potent antibiotics will generate a larger zone at lower antibiotic concentrations. In a variation of the Kirby-Bauer assay, thin strips containing calibrated gradients of antibiotics are marked with a scale, and the MIC can be read directly from the scale at the interface between bacterial growth and inhibition around the strip.

A second key concept in antimicrobial therapy is the distribution of the drug in the body. Antibiotics are compounds that can be used internally as well as externally. Antibiotics

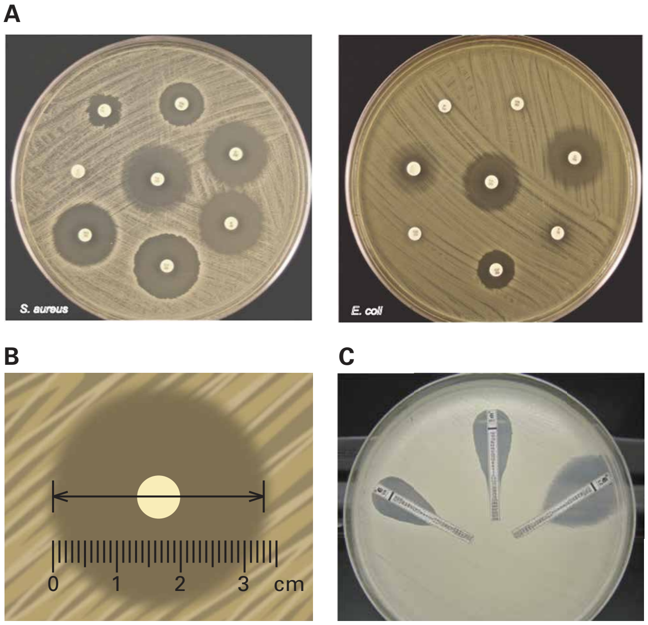


Figure 15-2. The Kirby-Bauer assay. The antibiotic susceptibility of a bacterium can be tested using an agar disc diffusion test. Filter-paper discs, each impregnated with a specified concentration of an antibiotic being tested, are placed on an agar plate spread uniformly with a culture of the bacteria. After incubation for a defined period of time to allow the bacteria to grow, the plate is examined for zones of inhibition, resulting from the antibiotic inhibiting the growth of the bacteria. The size of the cleared zone indicates the efficacy of the antibiotic to block bacterial growth. **(A)** Shown here are the results from testing strains of Gram-positive *Staphylococcus aureus* (left) and Gram-negative *Escherichia coli* (right). Courtesy of Tasha Sturm, Cabrillo College, with permission. **(B)** Zone of inhibition is usually measured as the length of the diameter in millimeters. **(C)** A *Staphylococcus aureus* isolate tested for antibiotic resistance by modified gradient diffusion assay using Etest strips with vancomycin (left), daptomycin (middle), and linezolid (right). The MIC of each agent is determined by the intersection of the growth with the strip, as measured using the scale inscribed on the strip. Reproduced from Jorgensen JH, Ferraro MJ. 2009. *Clin Infect Dis* **49:**1749–1755, with permission.

are not all equal inside the body, because each class of antibiotic has its own special **pharmacokinetics** that describe the distribution of the antibiotic in the body. For example, some of the antibiotics used to treat urinary tract infections (UTIs) concentrate primarily in the kidneys and urine and do not disseminate widely to other parts of the body. Others are readily absorbed from the gastrointestinal tract or bloodstream and permeate tissues all over the body. Obviously, the location of the bacteria responsible for causing an infection is key to selection of the appropriate antimicrobial compound. It does not help to treat a meningitis patient with a bactericidal antibiotic if the antibiotic does not cross the blood-brain barrier. In addition to initial distribution, the length of time an antibiotic remains in a location in the body before being degraded or removed is also important to understanding its efficacy. Considerable research time spent in the development of new antibiotics is focused on determining how these antibiotics are distributed and localized in the body and their duration in the body after they are administered.

A third key concept in antimicrobial therapy is side effects. Although the ideal antibacterial kills or inhibits the growth of bacteria specifically and does not affect mammalian cells (differential toxicity), such an ideal compound is not always available, and even an antibiotic such as penicillin, which comes very close to this ideal, can cause allergic reactions in some people. An example of side effects is the ability of some of the aminoglycoside antibiotics to destroy hearing in humans. Obviously, the patient's state of health and the seriousness of the infection influence the choice of appropriate antibiotic therapy. An aminoglycoside that causes deafness if administered long enough would not be considered for treatment of a relatively minor infection, but it might be the drug of choice for a critically ill patient whose infection can only be treated with this class of antibiotics. Unfortunately, because few new antibiotics against emerging multidrug-resistant bacterial pathogens, such as multidrug-resistant *Mycobacterium tuberculosis* (the causative agent of tuberculosis), are available, older antibiotics that were not widely used earlier because of serious side effects are increasingly being used as treatments of last resort. However, this strategy only makes treatment that much more difficult, because the long-term regimens required to treat tuberculosis, which involve the simultaneous administration of several different antibiotics, are tolerated by patients even less well than before.

All of these factors must be considered when choosing an effective therapeutic compound. In the remainder of this chapter, the focus will be on the effects of antimicrobials on the bacteria themselves, but these traits of antimicrobials and of the bacteria they target are only part of the treatment story. In the next chapter, we will discuss how bacteria become resistant to these antimicrobials.

Antiseptics and Disinfectants

Antiseptics and disinfectants, like antibiotics, are chemicals that kill or inhibit the growth of bacteria and other microorganisms. Most antiseptics and disinfectants are bactericidal. Most are also effective against many other types of disease-causing microbes such as viruses, fungi, and protozoa. This broad coverage has a drawback, however, because the chemicals used as antiseptics and disinfectants are often too toxic for internal use in humans and animals. Accordingly, they are applied only on inanimate surfaces or externally to skin or mucosal surfaces.

"Disinfectant" is the term used to describe antimicrobial compounds applied to inanimate objects and surfaces.

These can include harsh substances, such as chlorine compounds (e.g., bleach), reactive oxygen compounds (e.g., peroxides), iodine (e.g., Betadine), alcohols (e.g., isopropanol), phenolic compounds (e.g., carbolic acid, Lysol, triclosan, hexachlorophene) (see Box 15-1), cationic surfactants (e.g., quaternary ammonium compounds [QACs]), strong oxidizers (e.g., ozone), alkylating agents (e.g., formaldehyde), heavy metals and their salts, strong acids, and strong bases. Table 15-2 summarizes the mechanisms of action of these and other common disinfectants.

"Antiseptic" is the term used to describe antimicrobial compounds that can be applied to skin (e.g., handwashing preparations used in hospitals and doctors' offices and the waterless hand sanitizers that are now widely available in public locations). Antiseptics often include appropriately diluted or weaker versions of the above disinfectants, such as alcohol, hydrogen peroxide, iodine, chlorine, and quaternary ammonium compounds, or weak acids (e.g., vinegar, salicylic acid, sorbic acid). Other compounds, such as phenol or heavy metals, are too harsh or toxic for use on skin and are only used as disinfectants.

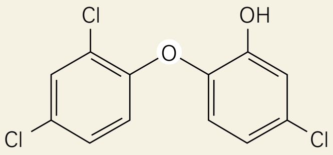
Antiseptics and disinfectants do best against actively replicating microorganisms. Bacterial spores are generally resistant to them, although the germination of spores can be inhibited. Otherwise, antiseptics and disinfectants are effective against a wide range of fungi, protozoa, viruses, and bacteria, and they tend to attack multiple targets in microbes. For example, **halides** such as chlorine or sodium

Box 15-1.
Triclo-Craze Hits the United States

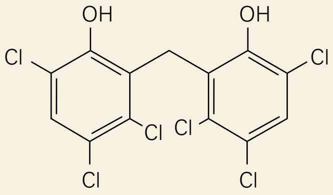
An antiseptic compound much in the news in the late 1990s was an antiseptic/disinfectant called triclosan. During the period under discussion, many companies began to market a variety of antibacterial plastic products, ranging from cutting boards to toys—products that are still popular with consumers today. This proved to be a very effective marketing strategy that sent millions of householders to their local stores to purchase these products that were guaranteed to protect them from deadly bacteria. The active ingredient in these products was a disinfectant/antiseptic called triclosan. Triclosan was not actually added to those products to make them safer. Instead, the plastics were impregnated with triclosan to prevent bacterial degradation of the plastics. Alert to the bacteriophobia of the times, however, advertising executives decided that they could sell products by touting the antibacterial compound they contained. The ads implied that these products contained a special additive other products did not have, when in fact virtually all plastic products contain it. On a scientific level, triclosan turned out to be unusual among disinfectants and antiseptics in that, unlike others, triclosan did have a specific target, an enzyme involved in fatty acid biosynthesis. A structural relative of triclosan (both are chlorinated bisphenols) is hexachlorophene, the active ingredient in many deodorants.

The popularity of products containing antibacterial compounds raised yet again the question of whether it is possible to be too clean. The human body evolved to accommodate massive microbial populations and regular contact with a variety of disease-causing microbes. The degree of sanitation and hygiene that has become the rule in modern times—at least in developed countries—has changed drastically and, in a short period of time, so has the degree of human exposure to disease-causing microbes. Some scientists have speculated that this change might be responsible for the rise in incidence of such conditions as asthma and inflammatory bowel disease, both of which are caused by a malfunctioning immune system. This rise has occurred primarily in countries that are noted for their high standards of hygiene. The argument is that an immune system that is not adequately challenged during childhood may become unbalanced or over-reactive to stimuli, leading to autoimmune reactions. If this view is correct, the incorporation of antibacterial compounds into soaps and other household products would only make the situation worse.

A Triclosan



B Hexachlorophene



Structures of triclosan **(A)** and hexachlorophene **(B)**. Triclosan is routinely added to plastics to prevent microbial degradation. Hexachlorophene is an antibacterial compound used in deodorants to prevent odor-producing activities of skin bacteria.

Table 15-2. Common disinfectants and their mode of killing

DISINFECTANT	MODE OF KILLING
Alcohols (ethanol, isopropanol)	Denature proteins
Alkylating agents (formaldehyde, ethylene oxide)	Form epoxide bridges that inactivate proteins
Halides (I^-, $C1^-$, $NaClO_4$)	Oxidizing agents
Heavy metals (Hg^{2+}, Ag^+)	Bind –SH groups, thus denaturing proteins
Phenols	Denature proteins; disrupt cell membranes by intercalating in them
QACs	Disrupt cell membranes by intercalating in them
UV radiation	Blocks DNA replication and transcription by damaging DNA

hypochlorite (household bleach) and iodine are strong oxidants that inactivate many bacterial proteins. **Hydrogen peroxide** has a similar mechanism of action. Halides and peroxide can also damage microbial DNA. **Quaternary ammonium compounds** (**QACs**) such as cetrimide and benzalkonium chlorides, which are some of the most widely used antimicrobial compounds, intercalate into phospholipid bilayer membranes, causing cells to leak vital ions and other small molecules. These compounds also disrupt electron transport chains.

Antibiotics

Characteristics of Antibiotics

Antibiotics are low- to medium-molecular-weight compounds that kill or inhibit the growth of bacteria and can be ingested or injected into humans and animals with minimal side effects. Table 15-3 provides a list of commonly used classes of antibiotics and their characteristics. Antibiotics can be either bactericidal or bacteriostatic. In contrast to most disinfectants and antiseptics, antibiotics generally interfere with a specific bacterial enzyme or process, such as the enzyme DNA gyrase that negatively supercoils the genomic DNA or the transpeptidase enzymes that cross-link peptidoglycan in the cell wall.

Several characteristics define a good antibiotic. First, the antibiotic must have few or no side effects. That is, it must be far more toxic to bacteria than to the human or animal body. This is known as the principle of **differential toxicity**. A second desirable characteristic, especially from the physician's perspective, is a **broad spectrum of activity** against many different types of bacteria. This is important because it is usually not possible to determine the identity of the bacterium causing an infection from the symptoms alone and clinical testing is often not convenient or expeditious. In addition, clinical testing is costly. Bacterial infections often have nonspecific symptoms, such as fever, malaise, and pus formation. Since it takes time to isolate and identify the bacterium responsible for an infection, it is useful to have antibiotics that are effective against the entire range of bacteria capable of producing a particular set of symptoms. Especially in the case of serious, rapidly progressing diseases such as bacterial pneumonia and septic shock, there is only a narrow window of efficacy and not much margin for error in the selection of an effective antibiotic.

Several other properties in addition to minimal side effects and maximum spectrum are required to make a good antibiotic. Antibiotics must have the appropriate **bioavailability** and **pharmacokinetics** to get to sites of infections. Bioavailability is a measurement of the rate or fraction of the drug that enters the systemic circulation and reaches the desired site of action. Pharmacokinetics is the tracking of the drug over time within the body, including its movement, distribution, metabolism, and excretion. For the same reasons that a broad-spectrum antibiotic is desirable, having excellent pharmacokinetics (uptake, distribution, and half-life properties) in humans or animals adds to the usefulness of the antibiotic. In addition, an antibiotic that can be absorbed from the digestive tract and administered in pill form is far easier to dispense than one that can only be administered intravenously by medical personnel. Last, cost becomes a serious consideration in antibiotic discovery and use. If an antibiotic is too costly to manufacture, it likely will not be developed by the pharmaceutical industry. Conversely, first-line antibiotics can be prohibitively expensive to use in developing countries.

Broad-spectrum antibiotics have an important drawback, which has caused scientists to take another look at this type of antibiotic. Such antibiotics not only attack the bacterium causing the infection but can also attack members of the resident microbiota of the body. Disruption of these commensal bacteria can allow other pathogens to cause infections. A timely example of this problem is *Clostridium difficile*, which is emerging as an extremely serious pathogen. *C. difficile* is normally a very minor member of the colon microbiota. Disruption of the normal colonic microbiota by antibiotic treatment allows *C. difficile* to overgrow and produce toxins that seriously damage the colon (see chapters 5 and 18). Because *C. difficile* forms hardy spores, it can be difficult to treat once established, and it can readily spread through diarrhea caused by the infection. A second example is yeast vaginitis in women who have taken antibiotics that disrupted their vaginal microbiota, thus allowing

Table 15-3. Antibiotics and their mechanisms of action

CLASS	MECHANISM OF ACTION	RESISTANCE MECHANISMS	SPECTRUM OF ACTIVITY	EXAMPLES COMMON NAMES
β-lactams (penicillins, cephalosporins, carbapenems, monobactams)	Inhibit transpeptidation step in peptidoglycan synthesis; bind penicillin-binding proteins; stimulate autolysins	Gram-negative outer membrane alterations; porin mutations; β-lactamase; modify target (alteration of penicillin-binding protein)	Gram-positive and/or Gram-negative bacteria (depends on agent)	Penicillin, Ampicillin, Cefobid, Augmentin (Amoxicillin + Clavulanic acid)
Glycopeptides	Inhibit transglycosylation and transpeptidation steps in peptidoglycan synthesis by binding D-Ala-D-Ala	Gram-negative outer membrane alterations; modify target (substitute D-Ala-D-lactate for D-Ala-D-Ala)	Most effective against Gram-positive bacteria	Vancomycin, Teicoplanin, Daptomycin, Ramoplanin, Mersacidin
Aminoglycosides	Bind 16S rRNA in 30S subunit of bacterial ribosome	Inactivation of antibiotic by adding groups; mutation in 16S rRNA genes	Broadly bactericidal	Kanamycin, Gentamicin, Streptomycin
Tetracyclines	Bind 16S rRNA in 30S subunit of bacterial ribosome	Inactivation of antibiotic; ribosome protection; mutation in 16S rRNA genes; efflux	Broadly bacteriostatic; some protozoa	Tetracycline, Doxycycline
Macrolides/ lincosamides	Bind 23S rRNA in 50S subunit of bacterial ribosome	Methylation of target; mutation in 23S rRNA genes; efflux	Bacteriostatic for most; bactericidal for some Gram-positive bacteria	Erythromycin (macrolide); Lincomycin, Clindamycin (lincosamides)
Streptogramins	Bind 23S rRNA in 50S subunit of bacterial ribosome	Inactivation of antibiotic by removing groups; mutation in 23S rRNA genes	Bacteriostatic individually; bactericidal in combination; used for multidrug-resistant enterococcal infections	Synercid (Dalfopristin + Quinupristin)
Fluoroquinolones	Bind DNA gyrase	Efflux; mutation in DNA gyrase; reduced uptake	Broadly bactericidal; can enter phagocytes to kill intracellular bacteria	Ciprofloxacin, Norfloxacin, Nalidixic acid
Diarylquinolines	Inhibit ATPase synthase proton pump	Efflux; loss-of-function mutation in PepQ peptidase	Anti-mycobacterial	Bedaquiline
Rifampin	Binds β subunit of bacterial RNA polymerase	Mutation in RNA polymerase	Broadly antibacterial; effective against mycobacteria	Rifampin (Rifadin, Rifampicin)
Trimethoprim/ sulfonamides	Inhibit enzymes responsible for tetrahydrofolate production	Mutations alter affinity of target enzymes	Broadly antibacterial; some fungi (*Pneumocystis jirovecii*), protozoa	Bactrim, Septra
Metronidazole	Nicks bacterial DNA and interferes with DNA replication	Decreased production of flavodoxin gene	Antibacterial (mainly anaerobes); antiprotozoal	Flagyl
Oxazolidinones	Bind 23S rRNA in 50S subunit of bacterial ribosome to block initiation of protein synthesis	Mutation in 23S rRNA genes	Bacteriostatic; broad spectrum against Gram-positive bacteria, mycobacteria	Zyvox

the yeast to overgrow and elicit an inflammatory response. Because of such experiences, greater consideration is now being given to the effect of new antibiotics on the resident microbiota, with the goal of minimizing the impact of the antibiotic on the beneficial microbiota.

Another problematic feature of broad-spectrum antibiotics is that, even if they do not disrupt the resident microbiota significantly, they may select for resistance to the antibiotic. Some bacteria in the resident microbiota are capable of causing serious infections if they escape from the area in which they normally reside. Members of the resident microbiota are a significant cause of hospital-acquired infections. An example is vancomycin-resistant enterococci (VRE) species. Enterococci are common inhabitants of the human colon. The widespread use (and overuse) of vancomycin to treat or prevent infections caused by *Staphylococcus aureus* and other Gram-positive pathogens has selected for VRE strains. These VREs are now wreaking havoc in some hospitals. Although enterococci are not highly virulent pathogens, when they do cause infection they can kill the patient if the infection is not brought under control quickly. Enterococcal infections caused by VRE strains kill nearly 40% of the patients who have them. We will discuss this topic more fully in chapter 18.

The Process of Antibiotic Discovery

To identify good antibiotic candidates, lead compounds are first subjected to numerous assays to evaluate their properties and potential activity. Compounds are often screened at a single fixed concentration to see if they inhibit the growth of bacteria that show sensitivity to a variety of antibiotics, such as *Streptococcus pneumoniae*. These whole-cell growth assays are then followed by MIC testing with a variety of bacterial species. Some compounds inhibit the growth of all species tested, whereas others inhibit only Gram-positive or Gram-negative species. Rarely, compounds are found that inhibit only one type of bacteria, such as the recently discovered diarylquinolines that inhibit only mycobacteria (see below). MIC testing can be coupled with determinations of whether compounds are bacteriostatic or bactericidal or bind strongly to carrier proteins in serum, as described above.

Compounds with favorable properties are then tested to see if they are cytotoxic to cultured eukaryotic cells or if they affect eukaryotic cell respiration. Compounds that pass these hurdles are next tested in rodent models to determine if the injected compounds reduce bacterial infections at distant sites or cause overt toxicity to the animals. Candidate compounds are then assayed in the bacteria-based **Ames test** to determine whether they can act as mutagens that damage DNA. The compounds may also be tested at this stage for resistance development in several key bacterial pathogens. If resistance develops at a low frequency (less than 10^{-7} resistant mutants per total colony-forming unit [CFU]), the genomes of resistant mutants are sequenced (see chapter 5) to locate possible targets or processes affected by the compounds. Compounds with favorable properties may then be assayed by a simple surrogate test using cultured mammalian epithelial cells, such as Caco-2 cells, to determine if the compound is absorbed by and passed through epithelial cells.

Finally, compounds that get this far are tested in two general kinds of systematic animal experiments. Pharmacokinetic analyses, including rates and levels of absorption, distribution, metabolism, and excretion (**ADME**) at different compound doses, are performed to determine what happens to the compounds once inside the animal hosts. Pharmacodynamic analyses are performed in parallel to learn what the compounds do to the animal hosts, including detecting any cytological changes, effects on organ functions, toxicities, and side effects.

Only after these types of data are acquired in model systems showing that the candidate compounds are effective and safe for possible use as new antibiotics can applications be filed with review boards and government agencies to carry out further testing, eventually in human volunteers. The review and approval process is outlined later in this chapter. Candidate compounds have to pass such a daunting gauntlet that it is little wonder that discovering new antibiotics is a difficult undertaking, despite its necessity.

Natural Products. Many existing antibiotics were obtained from bacteria or fungi that were isolated from soil and other environmental sources. These antibiotics are often referred to as **natural products**, to distinguish them from compounds that arise solely by chemical synthesis. Naturally occurring antibiotics are almost all products of secondary metabolism and are often produced in response to environmental stress or competition with other microbes. With the recent dearth of new antibiotics, there has been a resurgence of screening for new natural-product antibiotics from microbial and untapped eukaryotic sources, such as marine organisms. However, screening for antibiotic activity from natural products is challenging because the source material can vary from batch to batch and natural-product extracts are usually complex mixtures of chemicals that need to be fractionated to find active compounds. In addition, it should always be kept in mind that antibiotics synthesized by bacteria bring the complication that the producer species must have its own intrinsic mechanism of resistance. Therefore, operons imparting resistance to natural-product antibiotics are already out there in nature and can become a serious problem if they are genetically transferred from the producer bacteria to pathogens treated with the antibiotics.

The fact that bacteria produce antibiotics has raised the question of what the role of antibiotic production in nature is. An obvious explanation is that antibiotics are a kind of "germ warfare," in which the producing species use antimicrobial compounds to discourage microbial competitors. The problem with this widely accepted explanation is that antibiotic production by microbes growing in nature is so low that levels of antibiotics are undetectable under many conditions. An alternative explanation for the role of antibiotic production in nature is that bacteria use these compounds as signaling molecules. Although there is no definitive experimental basis yet for either the germ warfare theory or the signaling theory, there is growing evidence that bacteria use antibiotic-like compounds as signals. Recent studies indicate that antibiotics can modulate gene transcription and specific adaptive responses in bacteria in a dose-dependent manner, suggesting that these antibiotics may have a role in cell-to-cell communication.

Historically, the way compounds with desired properties were discovered and developed was by finding a "lead compound" in nature that exhibited desired antibiotic activity in the tests described above. However, these initial compounds almost always lacked sufficient activity or had unfavorable properties. The lead compound was then chemically modified into **semisynthetic antibiotics** that are more active, more stable, less toxic to hosts, or more capable of overriding bacterial resistance mechanisms. This process is referred to as building **structure-activity relationships (SARs)** and remains a keystone of drug discovery. The lead compound is chemically modified in numerous ways, and the biological properties of the modified compound sets are assayed using the battery of tests described above. Some chemical modifications improve antibiotic properties, whereas others have the opposite effect. The SAR is optimized until threshold goals of efficacy, spectrum, specificity, lack of toxicity, and bioavailability are reached. Of course, many compounds cannot be optimized for all of these properties and so fail as antibiotics for medical use.

One of the greatest challenges of natural products is their chemical complexity. The traditional semisynthetic route offered few ways to specifically modify the starting lead compounds through chemical synthesis. Therefore, there were a limited number of options for making chemical modifications in SARs, and the whole process remained dependent on biological production of the starting lead compound. Fortunately, some natural products, such as the penicillins, were simple and could be chemically synthesized completely or modified extensively. Recent advances in organic synthesis and semisynthetic methods have made the complete synthesis of complex natural products feasible in reasonably high yields. Therefore, extensive SAR strategies are becoming possible for natural-product antibiotics.

In addition to new synthetic approaches, much has been learned about the biosynthetic pathways used by natural producers to synthesize antibiotics, including complex polyketides belonging to the macrolide and tetracycline families of antibiotics. This knowledge is being applied to greatly increase yields of antibiotics by producer strains or by optimized genetically engineered surrogate strains. These biosynthetic pathways are likewise being genetically manipulated or even mixed among different organisms to produce new antibiotics with different structures and activities. Genome sequencing of microbes has led to the unexpected finding that putative clusters of genes for antibiotic biosynthesis are present in many bacteria, not just soil bacteria (such as *Actinomycetes* or *Streptomyces* species) that have traditionally been used as sources of antibiotics. Indeed, other soil species, such as *Flavobacterium* species, and even *M. tuberculosis* contain genes that may encode new types of antibiotics. Natural products from bacterial and eukaryotic sources remain a possibly rich source of new antibiotic compounds, as evidenced by the recent discovery of a potentially new class of antibiotic, named **teixobactin**, from a normally nonculturable soil bacterium. Teixobactin inhibits the growth of Gram-positive bacteria by binding to lipid II in peptidoglycan biosynthesis, as discussed in a later section.

Synthetic Antibiotics. Some antibiotics, such as the fluoroquinolones, are completely synthetic. That is, these antibiotics were derived by SAR studies from libraries of starting compounds that were synthesized by organic chemical methods. One might think that with the great chemical diversity out there, lots of organic molecules would be found that exhibit favorable antibiotic activity. This might be the case in theory, but in practice it has been difficult to find new synthetic antibiotics. So far, human ingenuity has not been nearly as effective as the biological process of evolution, which has had over 3 billion years to get things right and produce effective antibiotics.

There are many approaches being tried to identify or generate new antibiotics (see Figure 15-3). One of these is **combinatorial chemistry**. Starting with a particular chemical structure or scaffold, a large variety of chemical groups are added in a random fashion to create an array of new chemicals. This bank of compounds is usually then tested for inactivation of a particular bacterial target, such as a purified enzyme that plays an essential role in pathogenic bacteria, but lacks strong homologues in mammals. By using a core template structure and randomly screening a large bank of chemical groups to make new compounds, combinatorial chemistry changes the paradigm for discovery by introducing the notion that compounds can be synthesized as mixtures and then subsequently screened for inhibitory activities as mixtures.

When mixtures are produced in chemical reactions, the number of compounds produced increases exponentially. For example, in traditional chemical synthesis, a reaction joins two types of functional groups, A and B, to form a molecule A–B, which is then tested individually for activity. However, in a combinatorial approach, two of each functional group are included in a single reaction mixture to produce four product compounds: A1 + A2 + B1 + B2 = A1–B1 + A1–B2 + A2–B1 + A2–B2. If three of each group are included in the same reaction mixture, then nine compounds are produced, and so on. Each of the mixtures is then tested for activity, and if any one of the mixtures has the desired activity, the individual components within that mixture can be tested for activity to identify the specific component responsible for the observed activity.

By combining many components in these types of reactions, a very large number of mixtures (or **libraries**) can be made. The biggest advantage of combinatorial approaches is that the number of tests for the desired property can be greatly reduced if the libraries are used for testing instead of individual compounds. That is, it is much easier to perform an antibiotic screen on one mixture of 100 compounds

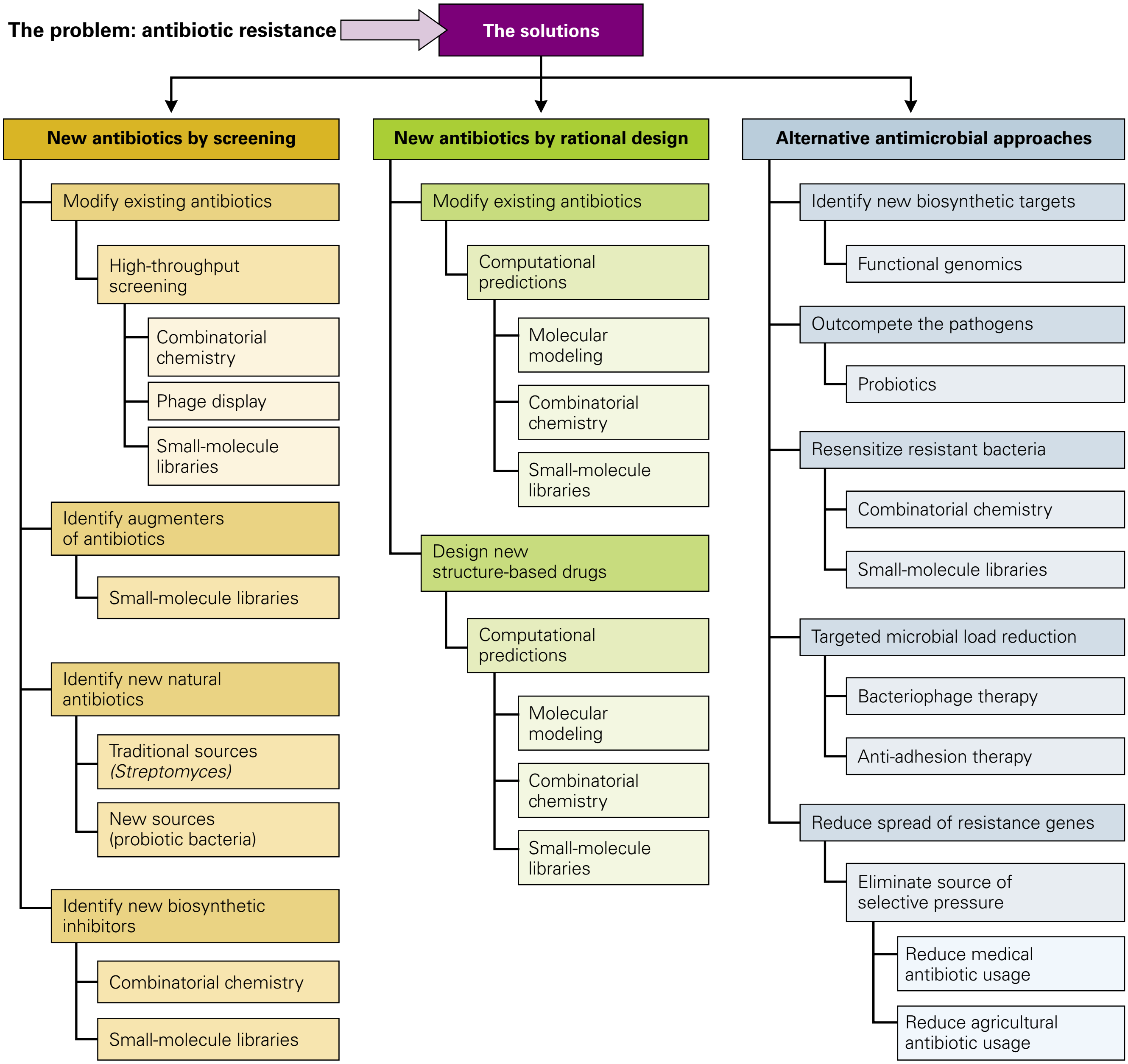

The problem: antibiotic resistance
The solutions
New antibiotics by screening
Modify existing antibiotics
High-throughput screening
Combinatorial chemistry
Phage display
Small-molecule libraries
Identify augmenters of antibiotics
Small-molecule libraries
Identify new natural antibiotics
Traditional sources (Streptomyces)
New sources (probiotic bacteria)
Identify new biosynthetic inhibitors
Combinatorial chemistry
Small-molecule libraries
New antibiotics by rational design
Modify existing antibiotics
Computational predictions
Molecular modeling
Combinatorial chemistry
Small-molecule libraries
Design new structure-based drugs
Computational predictions
Molecular modeling
Combinatorial chemistry
Small-molecule libraries
Alternative antimicrobial approaches
Identify new biosynthetic targets
Functional genomics
Outcompete the pathogens
Probiotics
Resensitize resistant bacteria
Combinatorial chemistry
Small-molecule libraries
Targeted microbial load reduction
Bacteriophage therapy
Anti-adhesion therapy
Reduce spread of resistance genes
Eliminate source of selective pressure
Reduce medical antibiotic usage
Reduce agricultural antibiotic usage

Figure 15-3. The search for new antimicrobials. The growing problem of antibiotic resistance has spurred the need for new antibiotics and alternative strategies to combat infectious bacteria. Solutions being pursued by biotechnology companies include refining and streamlining traditional approaches to discover new, or to modify existing, antibiotics as well as creating new designer drugs and exploiting genomics, probiotics, bacteriophages, and other alternative approaches. Adapted from Wilson BA, Salyers AA. 2002. *Curr Opin Biotechnol* **13:**267–274, with permission.

than separately on 100 compounds. The screen can then be dramatically ramped up and robotically automated to assay thousands of libraries, each of which consists of 100 or more compounds. This process, referred to as **high-throughput screening (HTS)**, enables the screening of hundreds of thousands of compounds in a relatively short period of time and uses considerably fewer chemical reagents and materials. Once it has been determined that a particular library has a desired activity or property, the problem changes to simply identifying which of the 100 compounds in that particular library mixture is the active one. The process of making that determination is called **deconvolution.**

A drawback of this combinatorial method is that because a purified target, such as an enzyme or rRNA molecule, is used in the HTS, it is not guaranteed that a compound that inactivates or inhibits the activity of that target will be able to get to the target in an intact bacterium. To get to an intracellular target, the antibiotic has to transit the outer membrane of a Gram-negative bacterium and the cytoplasmic membrane of both Gram-negative and Gram-positive bacteria. Also, the interior chemical milieu of the bacterial cell may not be conducive to antibiotic action. For these reasons, essential proteins on bacterial cell surfaces, such as enzymes involved in peptidoglycan biosynthesis, cell division, or signal transduction, are often chosen as targets. These targets are considered more **druggable** because antibiotics can reach them without traversing the cytoplasmic membrane. For this reason, most antibiotic discovery processes nowadays include at least one bacterial cell-based assay in the HTS process to determine target accessibility.

Another new approach is similar to the compound library HTS approach outlined earlier, except that scientists use the crystal structure of the target molecule as a guide to model the potential inhibitor into the binding sites of the target protein by using computer "docking" simulations and then synthesize chemicals that will bind to the target molecule and inactivate it. This approach is called **rational drug design** and was much heralded as the salvation of the pharmaceutical companies when it was first introduced. Rational drug design has some of the same drawbacks as combinatorial chemistry. More discouraging, however, is the fact that the results have been very disappointing. One explanation for this poor yield might be that the structures used as models are too rigid and do not take into account dynamic binding interactions that occur in solution, which might be needed to achieve higher affinity and specificity of the drug candidate. Another possible explanation might be that the computational tools used in the early stages of these studies were not as sophisticated and need further development to improve their predictive power.

A hybrid approach that combines library HTS with rational drug design seems to hold greater promise for finding synthetic antibiotics. In this approach, chemical libraries are screened for inhibitors of a purified bacterial target protein or RNA structure. The inhibitor is then co-crystallized with the target protein or RNA and the three-dimensional structure of the inhibited complex is determined by X-ray crystallography. Alternatively, for smaller targets, NMR structures of inhibited complexes can be determined. The three-dimensional structure of the inhibitor in the target is used as a starting point for further optimization of the inhibitor using more advanced computational modeling tools. The modified inhibitors are synthesized and tested for improved inhibition of the target. The tremendous advantage of this hybrid approach is that the modeling is driven by the structures of actual inhibitor complexes instead of a theoretical prediction of what molecules might bind to the target. However, even if strong inhibitors are synthesized, they still must be transported into cells for cytoplasmic targets and be amenable to further chemical modification to optimize all of the other properties of good antibiotics described previously.

Finding New Drug Targets by Exploiting Bacterial Genomics. As we shall see in the following sections, most of the clinically successful antibiotics act on a very short list of known cellular targets (i.e., a limited number of proteins and rRNA molecules involved in ribosomal function, DNA synthesis, or cell wall biosynthesis). Whole genome sequencing of hundreds of bacteria opened the prospect of identifying new targets for antibacterial drug discovery. The idea behind this **genomics-based discovery** approach was to compare the sequences of different pathogenic bacteria to find those genes that were highly conserved among them and consequently more likely to be potential broad-spectrum targets for drug development. The set of conserved genes could then be narrowed further to those that were unique to bacteria (or at least with low homology to any human counterpart) and that were essential for bacterial survival (i.e., would kill the bacteria if inhibited). The resulting targets would then be used in HTS to identify lead compounds for antibiotic development. There are currently many sizeable small-molecule library collections that have been assembled for HTS, each consisting of more than 200,000 compounds—and even some containing over a million.

With all of the bacterial genome sequences that have been made available over the past 10 years, you might ask how it is possible that so few new antibiotics have made it to the market. A number of explanations for this meager level of success have been put forward. Interestingly enough, it does not appear to be because of an inability to move potential targets to the HTS stage for lack of robust assays or because of the known challenge of converting a lead compound into a safe bioactive drug. It instead appears to be a combination of other factors, the primary one being the lack of sufficient molecular diversity in the nature of the compounds that make up the small-molecule libraries used for HTS. This lack of diversity does not allow for discovery of new structures with the desired activity or functionality.

The compound libraries are usually based on existing drugs or natural products and therefore consist of large numbers of compounds with similar structures or properties. Consequently, most of the compounds have particular structural features that are readily synthesized and pharmacological properties that are suitable for mammalian drug targets. That is, they are heavily biased toward compounds that follow **Lipinski's Rule of Five,** which is a medicinal chemist's empirical rule of thumb used to evaluate whether a potential lead compound has chemical properties that would make it a drug that is likely to be orally bioactive in humans without significant toxicity. Lipinski's rule states that a potential drug cannot violate more than one of the following criteria: it must have no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, a molecular weight under 500 Daltons, and an octanol-water partition coefficient (log P) of less than 5. Note that the numbers are all multiples of five, hence the rule's name.

The problem is that most known naturally occurring antibiotics do not follow Lipinski's rule. So, it looks like more chemical diversity, more inspiration from nature, and a better understanding of the physical and biochemical properties that are important for effective antibacterial activity are important if we are to move forward in discovering new antibiotics. The problem is compounded by the fact that most of these libraries are compendia of compounds and their derivatives that were initially screened against mammalian targets involved in other human diseases. Although the numbers of compounds in libraries sounds impressive, relatively few of the synthetic compounds have precedents as antibiotics.

Another factor was the lack of annotated bacterial gene targets with validated functions or activities, which precluded them from consideration for assay development in the first place. A surprisingly large percentage (approximately 25%) of the genes sequenced in each bacterial genome could not be annotated or could only be tentatively annotated based on weak homology to genes in other bacteria. It is impossible to develop a bioassay for a target protein of unknown function, and so by default a large portion of the genes could not even be tested as potential targets. Researchers also found that the putative targets identified through genomic comparisons as homologues of known genes in other bacteria did not always have the predicted function in the bacterial pathogens of interest. This was particularly a problem when the researchers were interested in more broad-spectrum antibiotics. In addition, it was found that many bacteria have considerable built-in redundancy, with more than one unrelated gene catalyzing the same reaction or having the same function, such that an inhibitor against one does not necessarily work against the other and so the efficacy or spectrum of the inhibitor is greatly reduced. One of the current challenges of bacterial physiology, genomics, and bioinformatics is to identify these genes of unknown function.

Finding New Antibiotics by Mining Microbial Genomes. As the number of microbial genomes has exponentially expanded, some researchers are realizing that the genomes of many microbes, particularly those belonging to families that are already known to produce antimicrobials, encode a wealth of uncharacterized natural-product biosynthetic gene clusters. These gene clusters include some genes that encode enzymes with known functions in secondary metabolism, as well as genes with unknown or poorly characterized functions that could introduce structural and/or activity diversity to any resulting metabolite (i.e., antibiotic). Consequently, researchers are eyeing these novel pathways as sources with enormous potential for novel antibiotic discovery and development.

From the thousands of genome studies conducted over the past decade, it appears that many microbes with large genomes, such as *Streptomyces* and other Actinobacteria, have the capacity to produce over tenfold the number of secondary metabolites as previously predicted from their observed metabolite profiles. For instance, the soil bacterium *Streptomyces rapamycinicus* has a 12.7-Mb genome, 3 Mb of which encodes 48 gene clusters predicted to encode diverse secondary metabolites, including the macrolide rapamycin (also known as sirolimus), an immunosuppressant drug used to prevent rejection of organ transplants. Many of the other gene clusters are cryptic and appear to encode novel secondary metabolites. However, next-generation genomic technologies coupled with advances in biochemical and analytical tools, such as high-throughput chromatographic separation plus mass spectrometry, for isolating and identifying the structures of metabolites present in cultures in minute quantities (a discovery approach coined "metabologenomics"), have made it possible to correlate the presence of those metabolites with the putative gene clusters that encode them. In addition, improved technologies for cloning the large gene clusters and for heterologous expression under constitutively active promoters in more genetically tractable bacterial systems have revolutionized the discovery and large-scale production and characterization process. See Box 15-2 for an interesting example of this metabologenomics approach that was used to discover a new bioactive natural product, tambromycin.

The Economics of Antibiotic Discovery

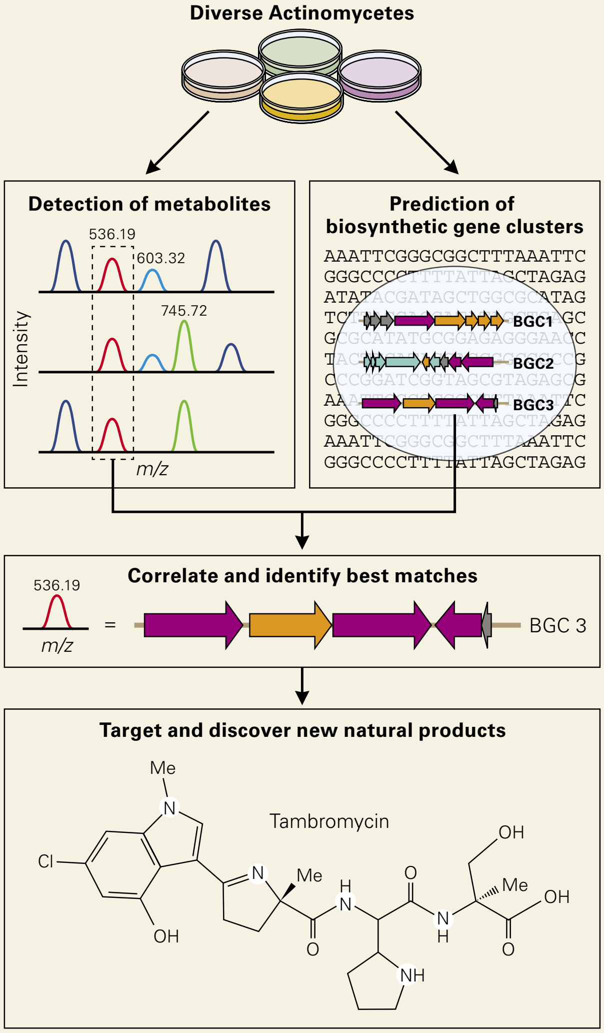
The earlier description makes it clear that antibiotic discovery and development are challenging processes with many hurdles. These processes are also costly and time-consuming. A company can spend $800 million or more to bring a new drug to market, and the process can take more than 10 years. At any point, complications, such as unfavorable side effects or insufficient efficacy in animals or human volunteers, may be uncovered, and the company loses its investment. Figure 15-4 shows an example of the process for development of a broad-spectrum antibacterial drug and the associated timelines and risks.

Why is bringing a drug from the research laboratory to market so expensive and time-consuming? First, the discovery and characterization processes require the efforts of a large team of highly paid scientists from many scientific disciplines, including microbiologists, medicinal organic chemists, structural biologists, and chemical modelers. At some stage, detailed pharmacokinetic and pharmacodynamic studies need to be performed in multiple animal species to evaluate efficacy and safety. A promising **investigational new drug (IND)** represents **intellectual property (IP)** and will have to be patented during these early stages, another element of the overall process that is both expensive and time-consuming.

Box 15-2.

Metabologenomics Discovery Approach to Identify New Bioactive Drug Candidates

Advances in genome sequencing technologies (genomics) have provided a cornucopia of genomic data that are now being mined with cutting-edge bioinformatics tools to identify shared gene clusters that encode putative biosynthetic enzymes involved in producing secondary metabolites. Improved detection and analytical tools such as liquid and/or gas chromatography coupled with mass spectrometry (LC-MS or GC-MS, respectively) enable detection and structure determination of the metabolites present in microbial culture mixtures (metabolomics). The marriage of these two approaches has enabled the large-scale discovery and characterization of potentially new bioactive compounds. A recent example of the application of this approach identified a novel peptide-derived bioactive natural product, tambromycin, from *Actinomycetes*. In this study, information obtained from 178 sequenced genomes of diverse *Actinomycetes* strains was used to find biosynthetic gene clusters (BGCs) that could be grouped into gene cluster families (GCFs) based on the metabolomics profiles generated using LC-MS data (accurate mass values, m/z) from the same set of bacterial strains. Pairwise correlation analysis then enabled the researchers to determine which metabolites were associated with which GCF. Ultimately, the researchers were able to identify tambromycin and its associated biosynthetic gene cluster. Hopefully, similar approaches will be used in the future to identify other bioactive natural product drug candidates.



Source:
Goering AW, McClure RA, Doroghazi JR, Albright JC, Haverland NA, Zhang Y, Ju KS, Thomson RJ, Metcalf WW, Kelleher NL. 2016. Metabologenomics: correlation of microbial gene clusters with metabolites drives discovery of a nonribosomal peptide with an unusual amino acid monomer. *ACS Cent Sci* **2:**99–108.

But what really contributes the most to the cost and time necessary to bring an IND to market is the eventual human clinical trials. Human clinical trials can only occur after data from extensive preclinical studies of the types described above are evaluated and approved by **institutional review boards (IRBs)** that are comprised of scientists, disease experts, statisticians, physicians, and bioethicists. Planning a clinical trial is an exceedingly complicated process that is beyond the scope of this book. But briefly, the study protocol must establish: dosing and administration regimens, patient recruitment and evaluation, criteria of efficacy and safety, logistics of trial implementation, and contingency plans for when to stop a failing trial. Large teams of physicians, statisticians, drug experts, and hospital administrators are required to conduct clinical trials, usually carried out at multiple institutions.

For antibiotic INDs, Phase I testing involves a relatively small number (between 20 and 100) of healthy adult volunteers to test for potential adverse side effects and safety. Phase II testing involves administering the drug to a moderately sized (between 100 and 500) population of volunteers with an infection that the IND antibiotic should treat. The efficacy and safety of the antibiotic are closely monitored, as is the health of the volunteers in the event that the trial needs to be stopped. Many drugs fail at Phase II because they are not efficacious enough in humans or cause unexpected side effects that were not seen in preclinical animal studies. Phase III trials are the most extensive and expensive of the testing paradigm. Phase III trials involve many more volunteers (usually thousands) with infections that are expected to be treatable by the antibiotic IND. They are usually set up using a **double-blind** protocol, in which the identity of the patients receiving the test drug, a comparator drug already on the market, and a placebo are unknown to participants or hospital staff during the trial. Phase III trials are designed to answer the critical question of whether the IND antibiotic is equal to or better than current treatments and whether side effects occur in a larger patient population. Only in the last stages of clinical testing are children and pregnant women included. Often two or more independent Phase III trials are completed for a particular infectious disease (also called an **indication**), and separate Phase II and III trials need to be carried out for each infectious disease that will eventually be on the drug approval label.

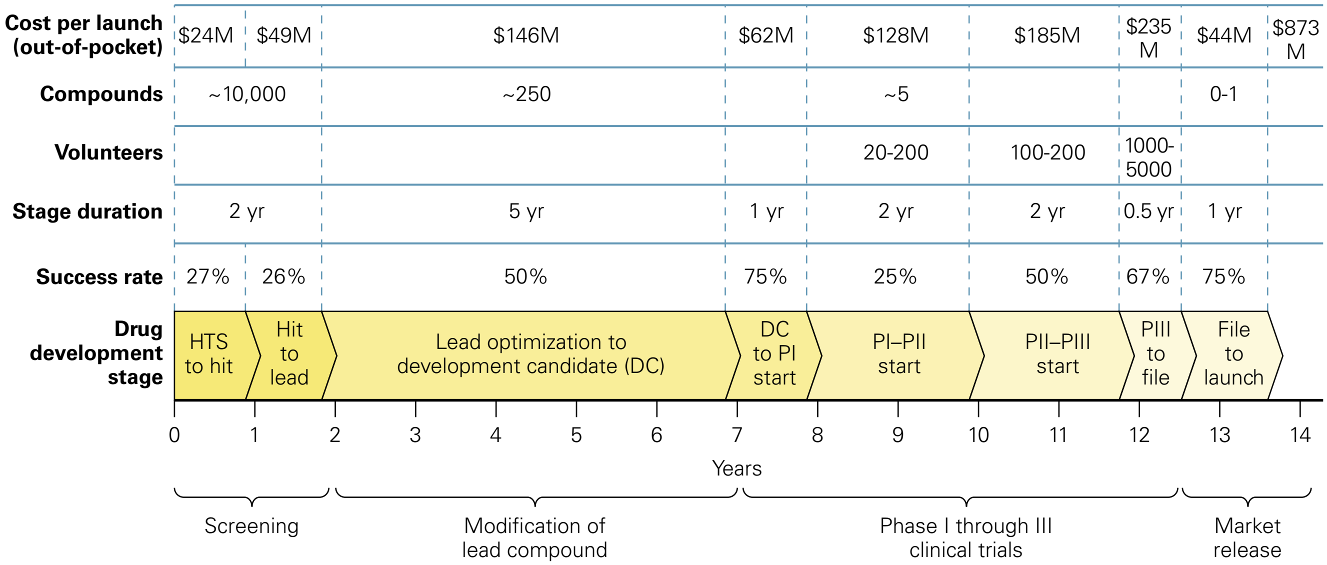
After the clinical trials are completed and the data are analyzed, the company presents its findings to the FDA for approval, a process that can take anywhere from months to years. The FDA is charged with ensuring that any drug released for use by the general public has passed a highly rigorous and thorough evaluation of safety and efficacy. Importantly, the FDA also requires that new drugs match or exceed those currently on the market in some way. Sometimes, restrictions must be put on the patient populations or types of uses approved for a particular drug. An antibiotic that is very effective but has some toxic side effects may be acceptable, if it is used as a last-ditch treatment for critically ill patients. However, it may not be approved for routine use or for administration to children. On the other hand, if a new IND antibiotic appears to be performing well in clinical trials, it could be administered to patients on a compassionate use basis (i.e., when the patient is critically ill and there are no other treatment options) even before the clinical trials are complete and the investigational drug is approved. The final stage, if FDA approval is obtained, is manufacturing and marketing (commercialization). Here again, there is a notable attrition rate, if a company determines that a new drug is not profitable enough to warrant production.

As new antibiotics became harder to find and more expensive to develop and test, pharmaceutical companies have become less enthusiastic about them. No antibiotic is going to be as profitable as simple synthetic drugs, such as Prozac or Viagra, that are taken repeatedly by many people over long periods of time. The result was that starting in the 1970s, when there was a glut of antibiotics on the market, the pharmaceutical companies started reducing or shutting down their antibiotic discovery programs based on marketing decisions. There was a small resurgence of antibiotic drug discovery starting in the late 1990s, when high-throughput screening, routine protein structure determination, and genomic information became available. However, at this writing, the situation is dire, and few of the larger pharmaceutical companies have retained antibiotic discovery groups large enough to have any chance of success. These developments have also negatively impacted antibiotic discovery by small biotechnology companies, which depend on attracting funding from larger pharmaceutical companies for their lead compounds or the biotechnology firm itself. While there have been a small number of success stories, many promising biotechnology companies focused on antibiotic discovery have folded.

But what about academic antibiotic discovery? New study panels have been established at funding agencies to encourage submission and review of grant proposals targeting antibiotic drug discovery and resistance development, and to foster HTS efforts. At this point in time, though, most government agencies are funding less than 15% of the investigator-initiated proposals submitted. This is an extremely low overall success rate. Also, in many ways these grant study panels have become as conservative as the pharmaceutical companies, because they fund research on known lead compounds rather than trying to find new kinds of lead compounds or supporting high-risk projects.

In addition, many academic studies identify inhibitors of bacterial growth or the activity of purified targets, but they often do not move to the next stages of establishing these inhibitors as true drug candidates by performing the assays for cytotoxicity, serum binding, mechanism of action in the bacterium, and bioavailability in mammals that were described above. Adding to this serious gap in approach, it is hard to assemble and maintain the multidisciplinary teams needed for drug discovery in academic institutions, especially using the short-term funding provided by many research grants. Finally, protection of intellectual property and rapid reporting of scientific advances in peer-reviewed journals, which are the lifeblood of companies and academic institutions, respectively, are seriously at odds. For these reasons, academic antibiotic discovery has only partially filled the void left by pharmaceutical companies abandoning antibiotic discovery and development.

This recent history has profound implications for public health. By the end of the 1980s, the flood of new antibiotics had slowed to a trickle (Figure 15-5). Moreover, bacteria are becoming increasingly resistant to antibiotics and are making some of the former antibiotic stars into has-beens. New antibiotics to cope with these resistant bacteria are now badly needed for an increasing number of diseases. But at the same time, the major players in the pharmaceutical industry have largely decimated their antibacterial research efforts. To add to this impending crisis, this chapter makes it clear that antibiotic discovery and development are complicated processes that take considerable time, effort, and resources (Figure 15-4). At this stage, even an abrupt resurgence in meaningful, concerted support of antibiotic discovery by the government, foundations, and



Drug development stage	HTS to hit	Hit to lead	Lead optimization to development candidate (DC)	DC to PI start	PI–PII start	PII–PIII start	PIII to file	File to launch	
Cost per launch (out-of-pocket)	$24M	$49M	$146M	$62M	$128M	$185M	$235 M	$44M	$873 M
Compounds	~10,000		~250		~5			0-1	
Volunteers					20-200	100-200	1000-5000		
Stage duration	2 yr		5 yr	1 yr	2 yr	2 yr	0.5 yr	1 yr	
Success rate	27%	26%	50%	75%	25%	50%	67%	75%	

Figure 15-4. The timelines and risks associated with broad-spectrum antibacterial drug discovery. Shown are the timelines and estimated success metrics for the development of a broad-spectrum antibacterial drug, based on the probability of success (percentages) metrics reported by GlaxoSmithKline and on clinical success rates based on industry averages reported by the Centre for Medicines Research. The most challenging, risky, and lengthy stage is lead optimization, which requires a sizable investment of time and medicinal chemistry effort to bring a lead found through high-throughput screening (HTS) to the beginning of Phase I (PI) clinical trials, and then again at the end of the Phase III (PIII) clinical trials, when filing a new drug for commercialization. Data from Payne DJ, Gwynn MN, Holmes DJ, Pompliano DL. 2007. *Nat Rev Drug Discov* **6**:29–40, and Paul SM, Mytelka DS, Dunwiddie CT, Persinger CC, Munos BH, Lindborg SR, Schacht AL. 2010. *Nat Rev Drug Discov* **9**:203–214.

industry would take years to have an impact before new antibiotics became available for use by the public. Nevertheless, such efforts are absolutely necessary to combat the growing threat of antibiotic-resistant bacterial pathogens to human health, as documented by a report from the Centers for Disease Control and Prevention (CDC) in 2013 and a national action plan issued by the White House in 2015.

Mechanisms of Antibiotic Action

Targets of Antibiotic Action

A drug that attacks a vital eukaryotic cell target will have serious side effects. Thus, the ideal antibiotic should act on a bacterial target that is either not present in eukaryotic cells or is different enough from the same molecule or process in

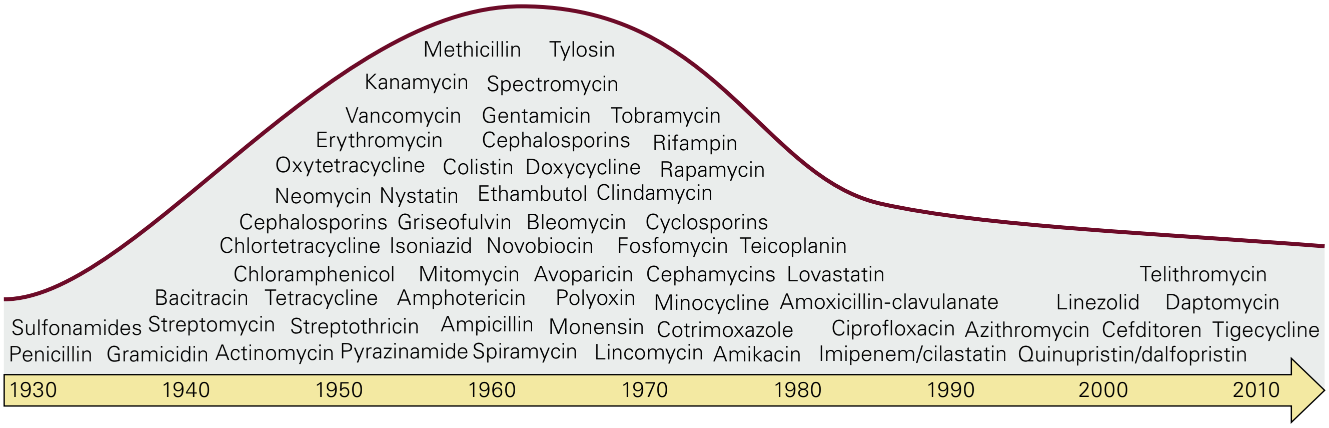
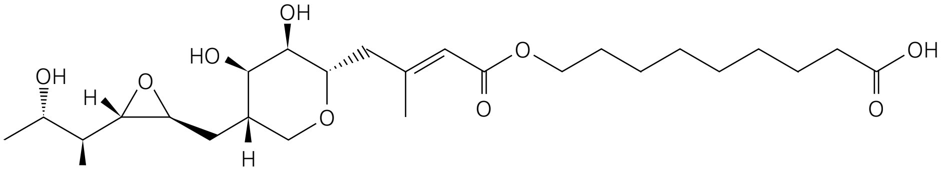


Figure 15-5. Timeline of antibiotic discovery.



Mupirocin

Figure 15-6. Structure of mupirocin. Produced by certain *Pseudomonas* species, mupirocin is a polyketide-derived antibiotic that targets bacterial tRNA synthetases.

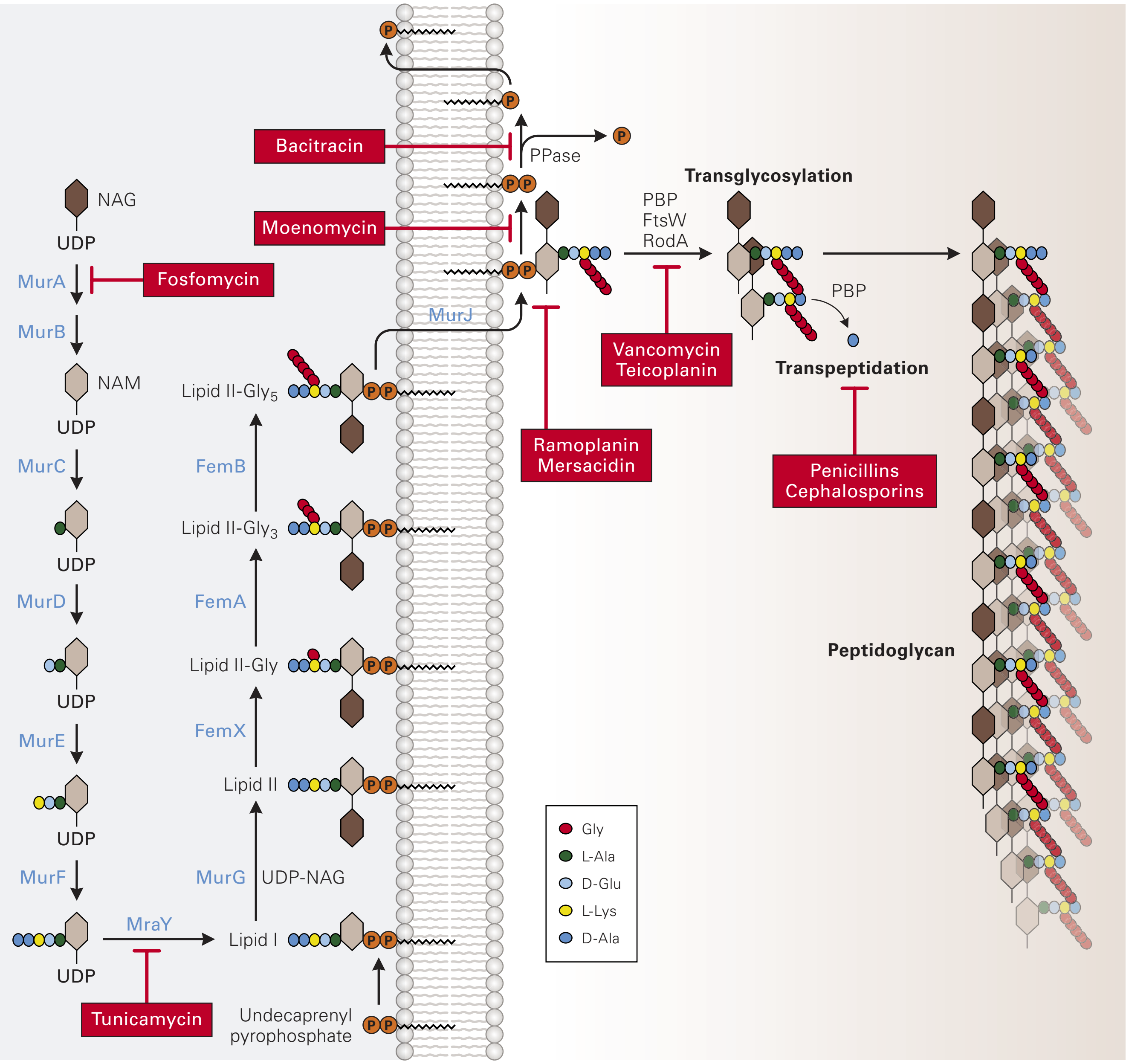
eukaryotic cells that there is little or no cross-reactivity. Although there are a number of targets that potentially satisfy this criterion, the currently used antibiotics tend to focus on a very limited set of targets. The most clinically relevant targets to date have been cell wall biosynthesis, protein synthesis, DNA synthesis, and folic acid synthesis. There are two main reasons why pharmaceutical companies have focused on these targets. First, in earlier screens of antibiotics, compounds that inhibited one of these processes emerged as the ones that were most effective and relatively nontoxic. Second, the expense of developing and testing a new antibiotic has become so high that administrators who approve funding for new antibiotic development feel more comfortable with drug classes and targets with which the pharmaceutical industry has had success in the past. Reaching out for new chemical classes of antibiotics that hit new targets runs the risk that the new antibiotics could fail at the clinical trial phase because of unexpected side effects and unexpected pharmacological properties. Hence, considerable effort has been expended using SARs and new chemical methods to improve antibiotic classes that already exist (the so-called "me-too, me-better" approach).

But even modification of known classes of antibiotics has its limitations. For example, the antibiotic mupirocin (Figure 15-6) is the only approved antibiotic that inhibits an aminoacyl tRNA synthetase (aaRS). Mupirocin is a polyketide-derived antibiotic originally isolated from *Pseudomonas fluorescens*. Mupirocin inhibits the isoleucyl-tRNA synthetase that attaches the amino acid isoleucine to its cognate tRNA^{Ile}. The isoleucyl-tRNA^{Ile} is subsequently used in translation, so mupirocin acts by ultimately blocking translation and inducing the stringent response. Mupirocin is bacteriostatic at low concentrations and bactericidal at high concentrations. It does not effectively inhibit the human isoleucyl-tRNA synthetase. Unfortunately, mupirocin can only be used topically in ointments on the skin and in the nose because it is rapidly metabolized and excreted by humans. Mupirocin has been used successfully to eliminate Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), from the noses of carriers. A hospital care worker who is a carrier of MRSA is a danger to patients who have surgical wounds, because *S. aureus* is a common cause of postsurgical infections. It is likely that the company that developed mupirocin performed extensive SARs to try to overcome its unfavorable pharmacokinetics in humans. However, these data are often considered proprietary and are seldom published. Similarly, it is difficult to know what SAR has been performed on other established antibiotics to see if they might be improved upon.

Cell Wall Synthesis Inhibitors

One unique feature found in bacteria and not in humans or animals is the bacterial cell wall. Not surprisingly, the bacterial cell wall has long been a target for antibiotic development because drugs that selectively target steps in cell wall biosynthesis do not act on eukaryotic processes and so are generally safe. An important component of the bacterial cell wall is the peptidoglycan (also called murein) that forms a meshlike matrix of polysaccharide chains cross-linked with short peptides (see Figure 2-5). The cell wall serves a structural role, holding the bacterial cell together and protecting against osmotic pressure. Blocking peptidoglycan biosynthesis interferes with the integrity of the cell wall, which can result in cell lysis, particularly during cell growth. Gram-positive bacteria have much thicker peptidoglycan layers than Gram-negative bacteria (20 to 80 nm versus 7 to 8 nm, respectively), and so they are often more sensitive to antibiotics that target the cell wall. Figure 15-7 provides an overview of the steps in peptidoglycan biosynthesis that are inhibited by different classes of antibiotics.

β-Lactam Antibiotics. The **β-lactam antibiotics** get their name from the four-membered β-lactam ring they all have in common. This group of antibiotics now includes four main types: **penicillins, cephalosporins, carbapenems,** and **monobactams** (Figure 15-8). β-lactam antibiotics have been among the most useful of all antibiotics. The main toxicity problem with these antibiotics is an allergic reaction that can occur due to formation of a β-lactam-serum protein conjugate that evokes an immune response due to the antibiotic acting as a hapten. Allergy to penicillins may result in allergy to cephalosporins as well, and vice versa. Fortunately, the single-ring-containing monobactams are different enough in structure from the double-ring-containing penicillins and cephalosporins that they can often be used on people who are allergic to penicillin.


NAG
UDP
MurA
Fosfomycin
MurB
NAM
UDP
MurC
UDP
MurD
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MurE
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MraY
Tunicamycin
Lipid I
MurG
UDP-NAG
Lipid II
FemX
Lipid II-Gly
FemA
Lipid II-Gly$_3$
FemB
Lipid II-Gly$_5$
Undecaprenyl pyrophosphate
MurJ
Bacitracin
Moenomycin
PPase
P
PP
Ramoplanin Mersacidin
Transglycosylation
PBP
FtsW
RodA
Vancomycin Teicoplanin
PBP
Transpeptidation
Penicillins Cephalosporins
Peptidoglycan
Gly
L-Ala
D-Glu
L-Lys
D-Ala

Figure 15-7. Steps in the biosynthesis of peptidoglycan and the effects of different classes of antibiotics. Peptidoglycan cell wall biosynthesis serves as the target for a number of antibiotics. Fosfomycin blocks the conversion of UDP-*N*-acetylglucosamine (UDP-NAG) to UDP-N-acetylmuramic acid (UDP-NAM). Tunicamycin inhibits the transfer of phosphor-NAM-pentapeptide from UMP to undecaprenyl phosphate. Bacitracin inhibits the dephosphorylation and recycling of the cell membrane lipid anchor, undecaprenyl (bactoprenol) pyrophosphate. β-lactams and glycopeptides, such as vancomycin and teicoplanin, block the transpeptidation step involved in cross-linking of the peptidoglycan layer. Because the enzymes that carry out transpeptidation also mediate transglycosylation, these antibiotics tend to block both steps. The moenomycin family of lipopolysaccharides and glycopeptides, such as mersacidin (particularly effective against MRSA), ramoplanin, and the macrocyclic depsipeptide, teixobactin, act primarily at the transglycosylation step. Enzymes involved in each of the steps are shown in blue text. PBP, penicillin-binding proteins. Data from Pinho MG, Kjos M, Veening JW. 2013. *Nat Rev Microbiol* **11:**601–614.

β-lactam antibiotics kill bacteria by inhibiting the last step in peptidoglycan synthesis, the transpeptidation reaction that cross-links the peptide side chains of the polysaccharide peptidoglycan backbone (Figure 15-7). β-lactam antibiotics also bind to and inhibit the action of other inner membrane proteins that may have a role in peptidoglycan synthesis. Transpeptidase and these other proteins are sometimes called **penicillin-binding proteins**. β-lactam antibiotics work by forming a covalent bond with a reactive serine residue in the active site of the transpeptidase (Figure 15-8).

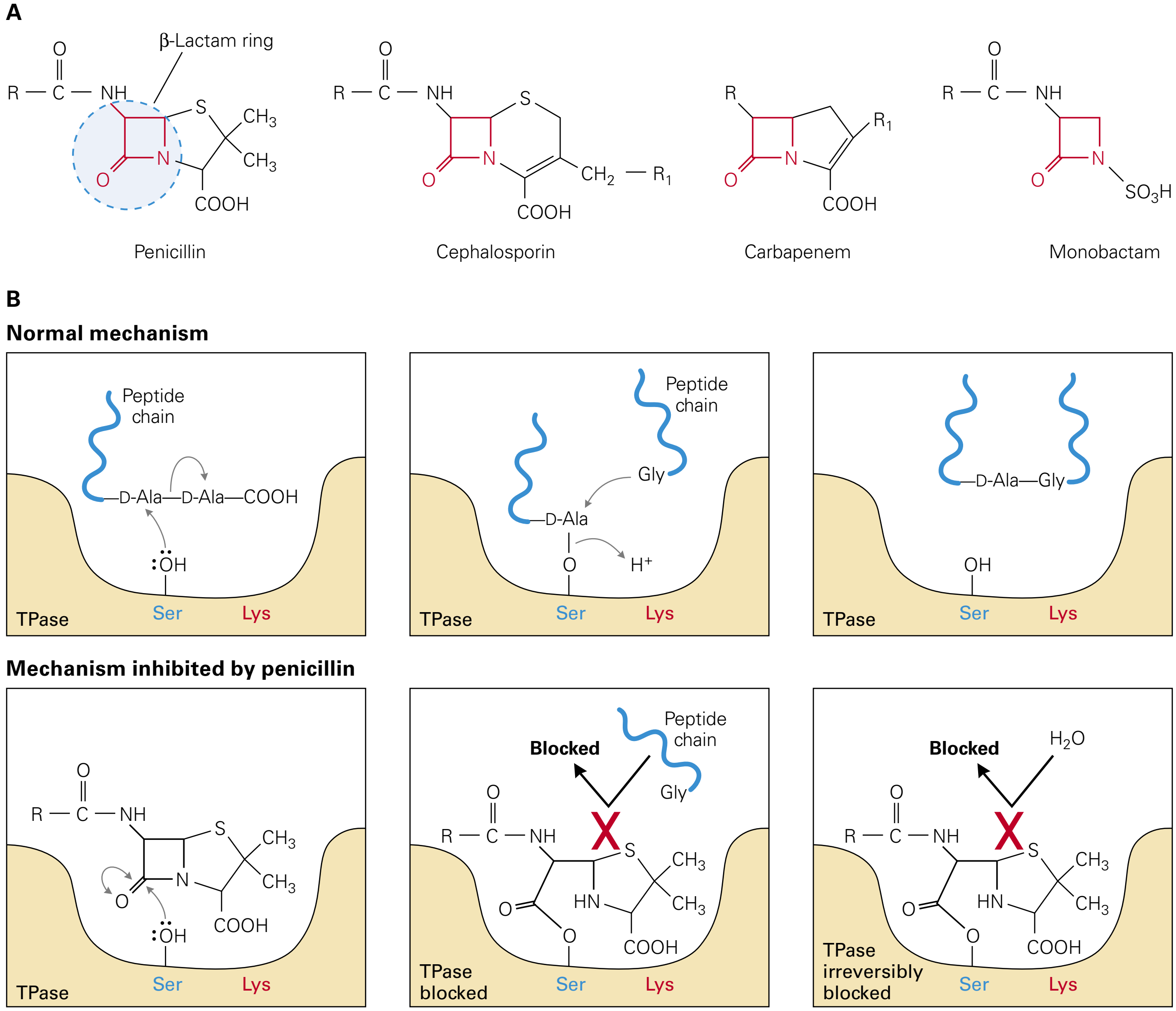

A
β-Lactam ring
Penicillin
Cephalosporin
Carbapenem
Monobactam
B
Normal mechanism
Peptide chain
D-Ala—D-Ala—COOH
:OH
Gly
D-Ala
H+
D-Ala—Gly
OH
TPase
Ser
Lys
Mechanism inhibited by penicillin
Blocked
H2O
TPase blocked
TPase irreversibly blocked

Figure 15-8. Structure of common β-lactam antibiotics and their mechanism of action. (A) The core structures of representative β-lactam antibiotics are shown. The four-membered β-lactam is strained and therefore subject to nucleophilic attack. **(B)** The mechanism by which β-lactam antibiotics bind covalently to and inhibit transpeptidase (penicillin-binding protein) enzymes (TPase). A reactive serine in the TPase active site that normally attacks the D-Ala-D-Ala group of the peptidylglycan chain to catalyze the transpeptidation reaction (top panel) instead binds the β-lactam antibiotic and opens the β-lactam ring (bottom panel). Water is not allowed into the active site to release the inactivated antibiotic from the covalently bound TPase enzyme.

This reaction opens the β-lactam ring and forms a covalent adduct that inactivates the enzyme. Notably, the covalently modified transpeptidase active site prevents water from freeing the serine residue, such that the resulting covalent complexes are unusually stable.

Inhibition of cross-bridge formation by β-lactam binding also triggers endogenous enzymes called **autolysins** that degrade the peptidoglycan. Normally, these enzymes function in turnover of peptidoglycan that allows orderly growth and division of the bacteria. The action of β-lactam antibiotics apparently removes controls that normally keep these enzymes in check and stimulates their attack on peptidoglycan. The peptidoglycan cell wall prevents the bacteria from bursting due to the high turgor pressure of the cytoplasmic contents relative to the external medium; therefore, weakening the cell wall leads to bacterial lysis, particularly during the log phase of cell growth. β-lactam antibiotics are normally bactericidal. Occasionally, if bacteria are in a high osmolarity compartment of the body (kidney) or if environmental conditions, such as pH, prevent

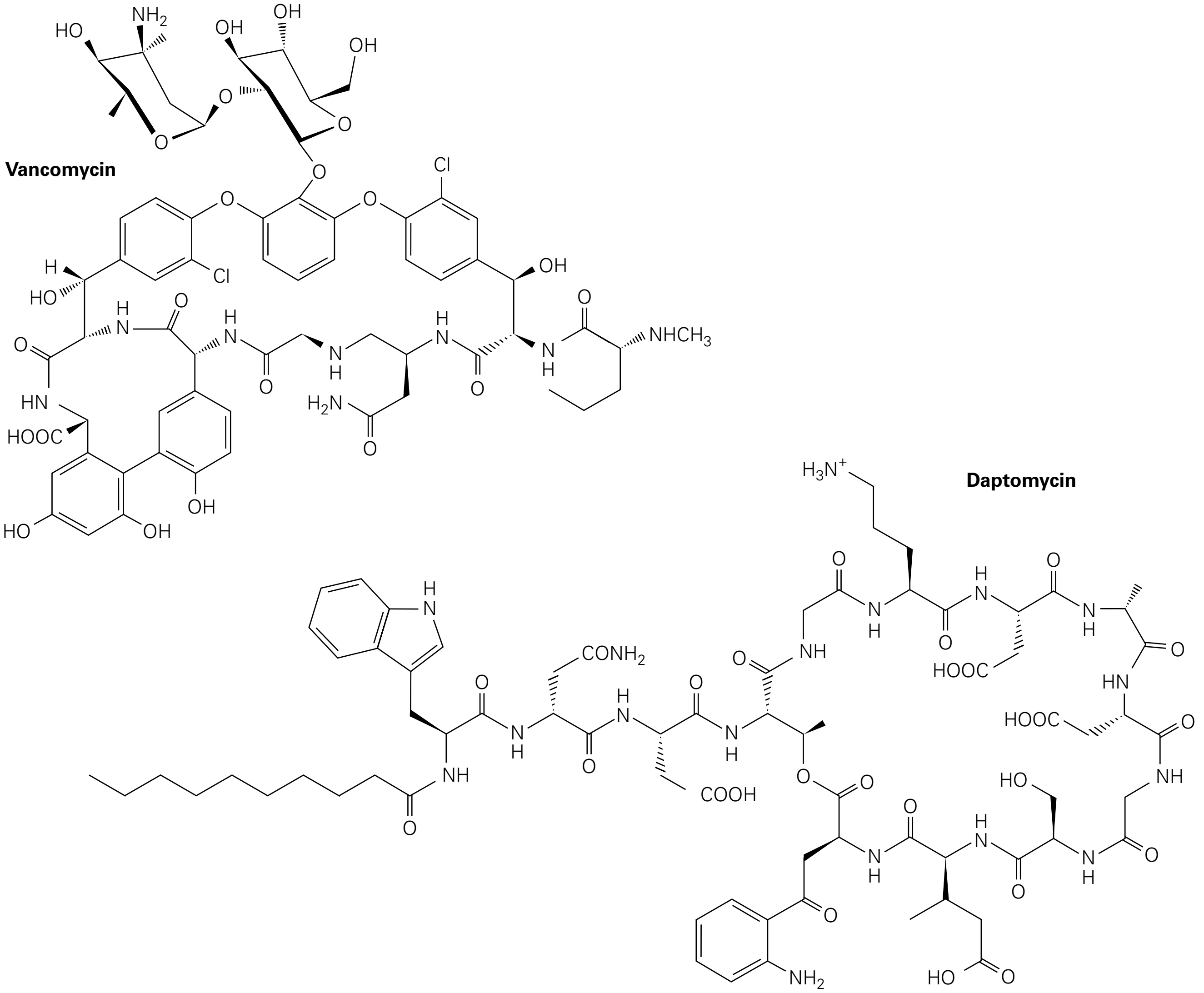


Figure 15-9. Structure of the cyclic peptide-derived antibiotics vancomycin and daptomycin. Vancomycin is a cyclic glycopeptide antibiotic produced by *Amycolatopsis orientalis*. Daptomycin is a lipopeptide antibiotic produced by *Streptomyces roseosporus*.

activation of the autolysins, bacteria can sometimes escape the killing effects of β-lactam antibiotics. Luckily, such cases are uncommon.

Different β-lactams differ in their spectrum of activity. Some are effective against both Gram-positive and Gram-negative bacteria, whereas others are much more effective against Gram-positives than Gram-negatives, or vice versa. Many thousands of β-lactam derivatives have been synthesized and characterized in SAR studies. Despite all of the time and money invested to date in studying β-lactam antibiotics, it is still not possible to predict with certainty what changes in the basic β-lactam structure will produce a more effective antibiotic. β-lactams also differ widely in their toxicity, stability in the human body, rate of clearance from blood, whether they can be taken orally, and their ability to penetrate the blood-brain barrier. But we seem to be approaching a limit to how much we can change β-lactam antibiotics to improve them as antibiotics that evade resistance mechanisms.

Glycopeptides. Another group of peptidoglycan synthesis inhibitors is the **glycopeptides** and/or **lipopeptides**, which include **vancomycin**, **daptomycin**, and **teicoplanin**. These antibiotics, especially vancomycin and more recently daptomycin (Figure 15-9), have become extremely important medically because they are the last drugs that are effective against some Gram-positive pathogens such as *S. aureus* and *Enterococcus* species. Teicoplanin is a semisynthetic glycopeptides antibiotic with activity similar to vancomycin, in which the fatty acyl side chains that are attached to the

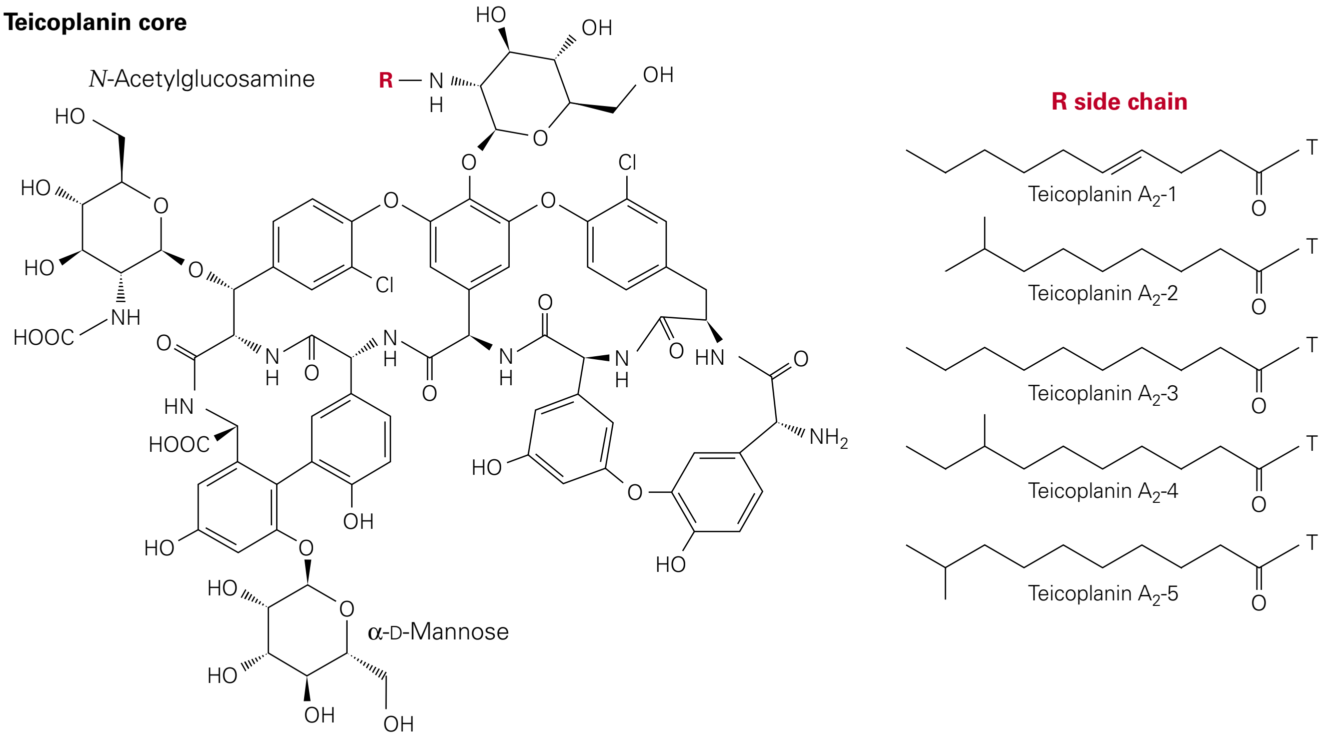
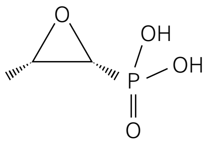


Figure 15-10. Semisynthetic side-chain modifications of the cyclic peptide-derived antibiotic teicoplanin. Produced by *Actinoplanes teichomyceticus*, teicoplanin has a cyclic glycopeptide core with different lipid side chains (R).

glycopeptides core structure differ among the compounds (Figure 15-10).

Vancomycin and teicoplanin bind to the D-Ala-D-Ala portion of the UDP-muramyl-pentapeptide after it is transferred out of the cell cytoplasm. Binding appears to inhibit **transglycosylation** and **transpeptidation**, the two final steps in peptidoglycan synthesis. Vancomycin and teicoplanin are used primarily to treat infections caused by Gram-positive bacteria and are not very effective against Gram-negatives because they cannot penetrate the outer membrane. Despite their relatively narrow spectrum, these antibiotics are clinically important. For example, vancomycin has become particularly important for treatment of infections due to strains of *S. aureus* that are resistant to virtually all other antibiotics.



Fosfomycin

Figure 15-11. Structure of fosfomycin. A metabolite from the *Streptomyces* species, fosfomycin contains a reactive epoxide ring, which is a potent electrophile that can be attacked by many nucleophiles (hydroxyls, amines, and thiols), and an unusual carbon-phosphorus bond. Fosfomycin enters bacterial cells via the glycerophosphate transporter and acts as a phosphoenolpyruvate (PEP) analog that inhibits the enzyme MurA in peptidoglycan biosynthesis by alkylating an active site Cys residue.

Daptomycin has a different mechanism of action from the other glycopeptides antibiotics; it inserts into the bacterial cell membrane of Gram-positive bacteria and aggregates to create holes that leak ions and cause membrane depolarization. Daptomycin was originally discovered and developed by researchers at Eli Lilly in the 1980s. It showed promise in Phase I and II clinical trials in the 1980s for the treatment of Gram-positive infections, but it was found to have adverse side effects on skeletal muscle and was then dropped by the company. Cubist Pharmaceuticals subsequently acquired the rights to the compound in 1997 and the drug was reintroduced on the market in 2003, but with a different treatment regimen that reduced toxicity to acceptable levels. In 2015, Cubist Pharmaceuticals was purchased by Merck, which quickly terminated its antibiotic discovery efforts.

Other Antibiotics That Inhibit Cell Wall Synthesis. **Fosfomycin** (Figure 15-11) and **bacitracin** (Figure 15-12) are two antibiotics that inhibit peptidoglycan synthesis by inhibiting different steps in the pathway than those inhibited by β-lactams and glycopeptides. Fosfomycin acts as a

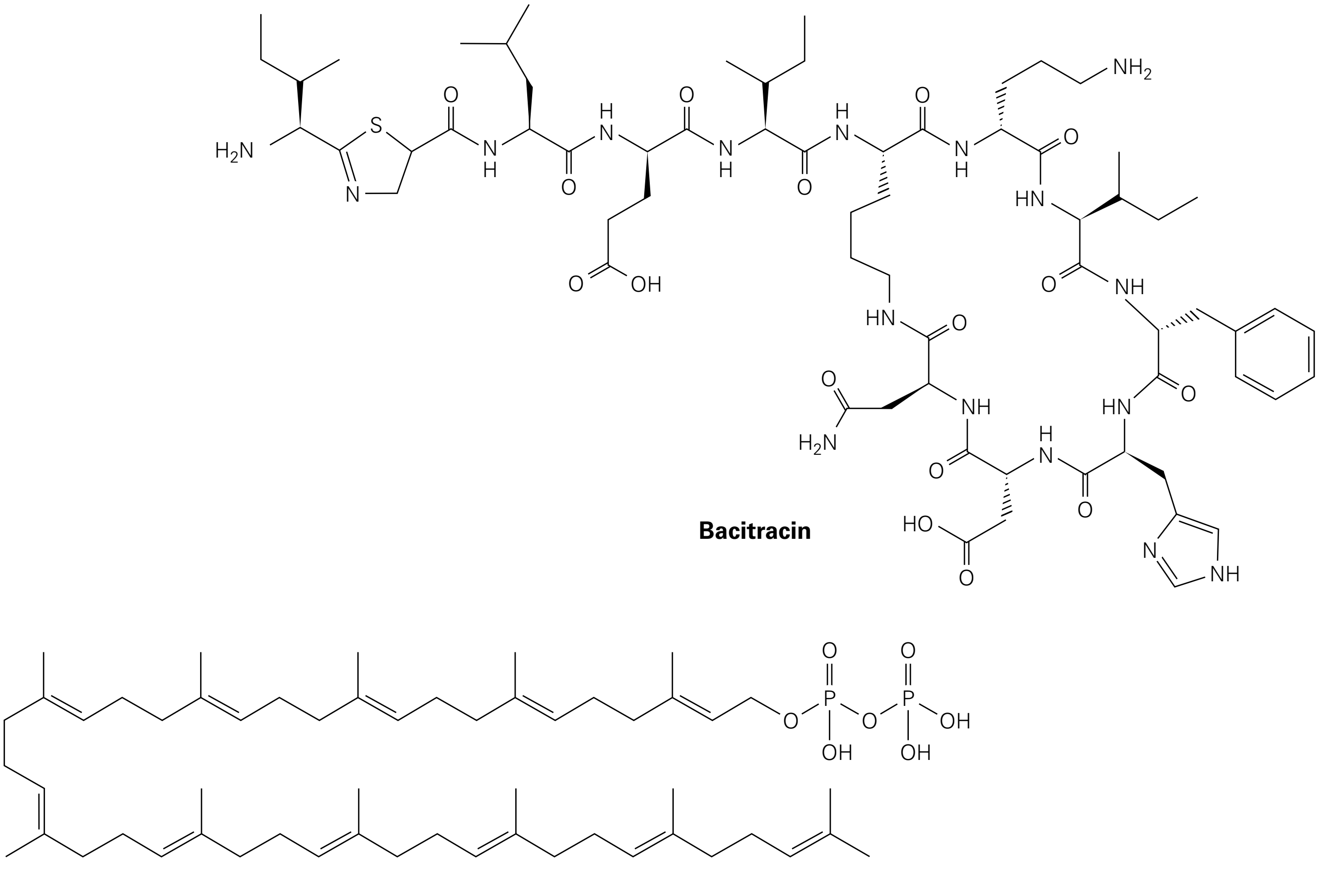


Figure 15-12. Structure of bacitracin. A cyclic polypeptide produced by certain *Bacillus* species, bacitracin inhibits the recycling of undecaprenyl (bactoprenol) pyrophosphate.

mechanism-based inhibitor (also known as a suicide substrate or "warhead") of the enzyme MurA, which catalyzes the conversion of UDP-N-acetylglucosamine (UDP-NAG) to UDP-N-acetylmuramic acid (UDP-NAM) in the first step of the pathway (Figure 15-7). The epoxy ring of the compound covalently reacts with a cysteine side chain in the enzyme's active site, thereby irreversibly inactivating the enzyme. Fosfomycin has limited clinical utility due to its toxicity inside the body, but it is used externally to treat UTIs and MRSA.

Bacitracin (Figure 15-12) interferes with recycling of undecaprenyl (bactoprenol) pyrophosphate, which is the membrane carrier lipid involved in peptidoglycan cell wall biosynthesis. Bacitracin inhibits the pyrophosphatase (PP_iase) that normally converts undecaprenyl pyrophosphate to undecaprenyl phosphate so that it can be returned to the cytoplasmic side of the membrane to act as a carrier again (Figure 15-7). It is very effective against Gram-positive bacterial cell walls. Bacitracin is used primarily in topical ointments available over the counter for treating a variety of skin infections, as well as preventing wound infections, but it is too toxic for internal use.

Protein Synthesis Inhibitors

One of the most significant discoveries in structural molecular biology occurred when the three-dimensional structures of ribosomes were determined. These elegant structures revealed that it was the rRNA molecules that catalyzed peptide-bond formation instead of the many ribosomal proteins. In addition to having profound implications for evolution and the mechanisms of translation, these structures have led to a deeper understanding of how some classes of antibiotics block bacterial translation (Figure 15-13).

Most protein translation inhibitors are large natural products with side groups that can form multiple hydrogen bonds and salt bridges. It turns out that these antibiotics inhibit translation by forming spatially specific hydrogen bonds to the bases and phosphate backbones of the 16S and 23S rRNAs in the ribosome functional sites. In addition, ribosome structures have revealed differences between prokaryotic and eukaryotic ribosomes that impart the differential selectivity. This knowledge is driving new efforts to modify older antibiotics to improve their binding affinity and selectivity, as well as to discover chemicals that bind to bacterial rRNAs in new ways.

Aminoglycosides. **Aminoglycosides** are trisaccharides with amino groups that act by binding tightly to specific sites in the 16S rRNA in the 30S subunit of the bacterial ribosome, thereby blocking protein synthesis (Figure 15-13). Binding of aminoglycosides, such as **kanamycin** and **gentamicin** (Figure 15-14), to the bacterial ribosome does not prevent the 30S subunit from binding mRNA and placing an fMet-tRNA in the P site, but it does block the 50S subunit from joining the 30S to form the active ribosome. Because protein synthesis is essential for continued viability of a bacterium, aminoglycosides are bacteriostatic or bactericidal, depending on their concentration. Aminoglycosides are effective against a number of pathogenic bacteria, but they have serious side effects that limit their use. Prolonged use of aminoglycosides can lead to hearing loss and impairment of kidney function.

Tetracyclines. Another class of antibiotics that targets the bacterial ribosome and binds to the 16S rRNA in the 30S subunit is the tetracyclines. **Tetracyclines**, as the name suggests, are compounds consisting of four fused cyclic six-membered rings (Figure 15-15). The effect of binding is to distort the A site and prevent the alignment of aminoacylated tRNA with the cognate codon on mRNA. **Tigecycline**, a tetracycline derivative that has a bulky glycyl-glycine side

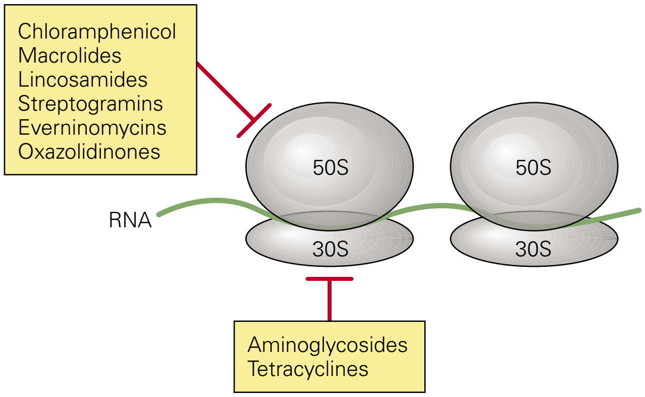
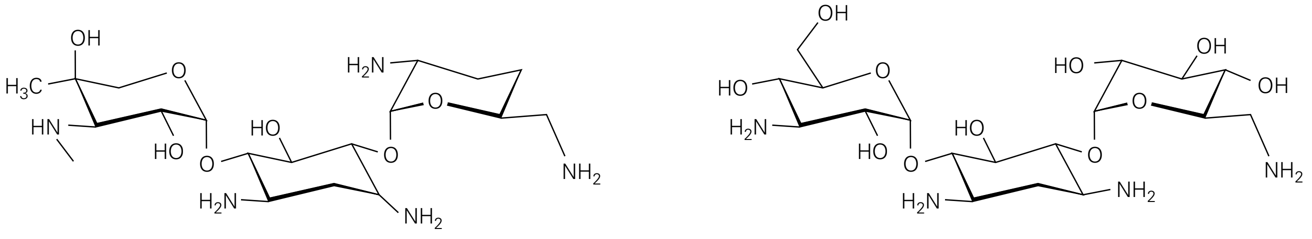


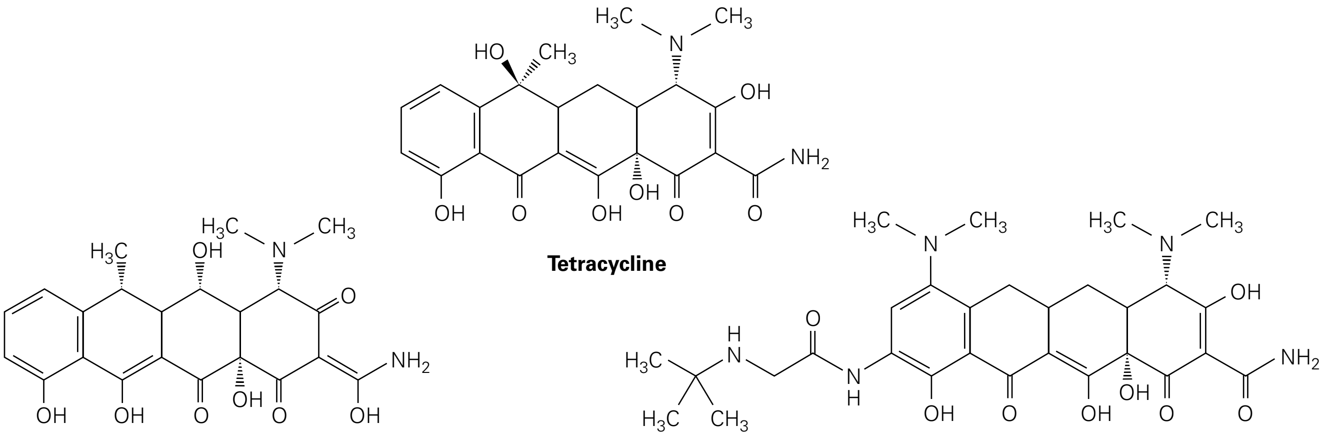
Figure 15-13. The bacterial ribosome as a target for antibiotics that interfere with protein synthesis.



Gentamicin

Kanamycin

Figure 15-14. Structure of the aminoglycosides gentamicin and kanamycin. Gentamicin is produced by Gram-positive *Micromonospora* species. Kanamycin is produced by *Streptomyces kanamyceticus*.



Tetracycline

Doxycycline

Tigecycline

Figure 15-15. Structure of tetracyclines. Tetracycline is a polyketide-derived antibiotic produced by *Streptomyces* species. There are also semisynthetic tetracycline derivatives available, such as tigecycline and doxycycline with improved properties.

chain, is a bacteriostatic antibiotic that can be administered intravenously for use against a number of both Gram-negative and Gram-positive pathogens. **Doxycycline**, which can be administered orally or intravenously, is an inexpensive generic drug that is a mainstay for health care systems in developing countries.

Although tetracycline can cause discoloration of teeth if given to young children or pregnant women and sometimes causes nausea or phototoxicity (sensitivity to light exposure), it is one of the least toxic antibiotics ever produced. This, together with the fact that it can be given orally, has led to overuse of tetracycline in clinical practice. Tetracycline has also been used in the treatment of acne and as a feed additive to promote growth of livestock. Not too surprisingly, tetracycline resistance is now so widespread that the utility of the tetracycline family has been considerably diminished. Nonetheless, tetracyclines still have some important uses, especially in the treatment of Lyme disease and some sexually transmitted bacterial diseases such as chlamydia and gonorrhea. Dermatologists still use tetracycline extensively to treat acne and rosacea (excess reddening of the skin of the face).

Scientists would like to find new members of the tetracycline family because this family of antibiotics has many good properties. It has been so widely used (and abused) precisely because it is not only effective but also nontoxic. Most tetracyclines unquestionably act by interfering with protein synthesis, but some members of the tetracycline family (e.g., **chelocardin**) appear to act instead by disrupting the bacterial membrane, not by stopping protein synthesis. At present, most of these atypical tetracyclines are of academic interest only, because they have so far proven to be too toxic for use in humans. There is hope, however, that some nontoxic derivatives can be found.

Macrolides. The **macrolide** family, exemplified by **erythromycin** (Figure 15-16), is another group of polyketide-derived antibiotics with relatively few side effects. Macrolides inhibit bacterial protein synthesis by binding the 23S rRNA in the 50S ribosomal subunit (Figure 15-13). Binding of macrolides blocks the tunnel used by nascent peptides to exit the ribosome. This is a particularly striking example of an antibiotic throwing a monkey wrench into the ribosome molecular machine. Macrolides are bacteriostatic for most

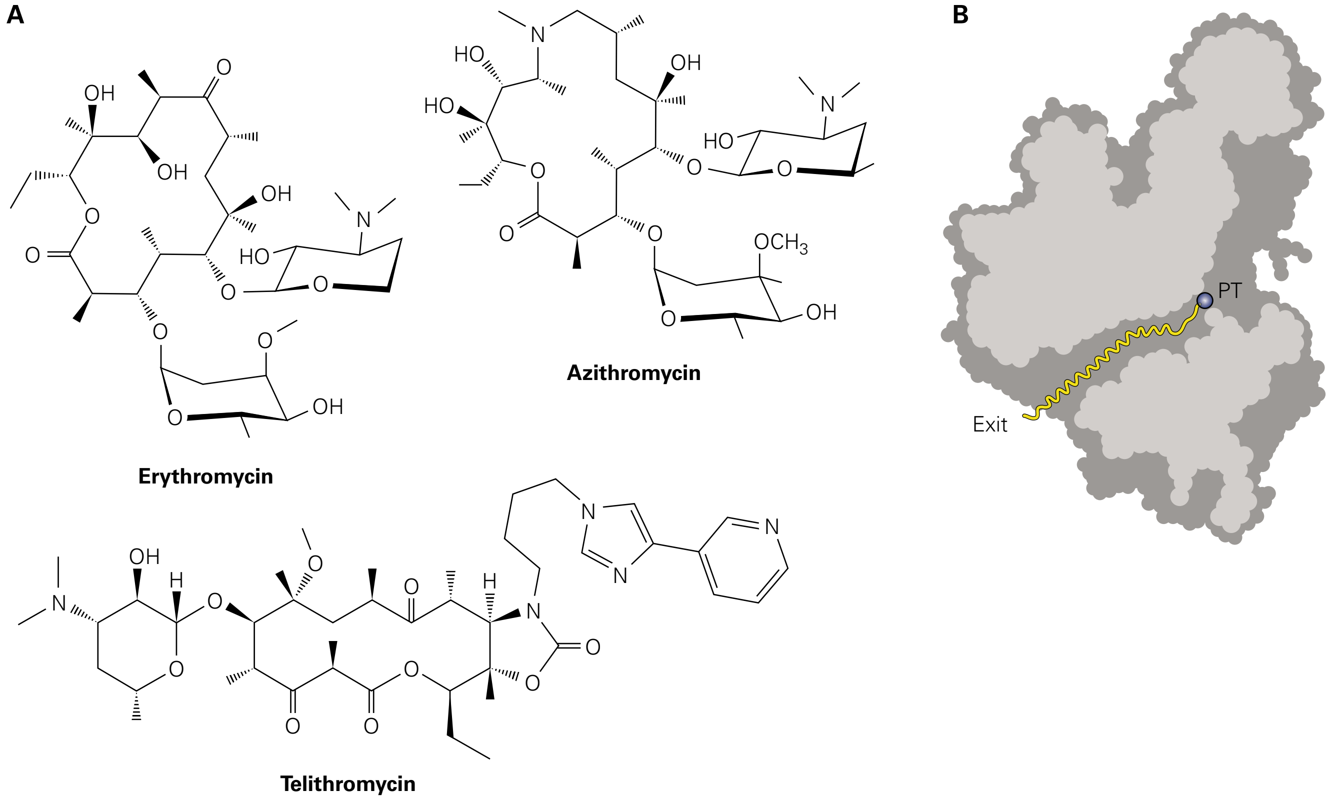


Figure 15-16. **Polyketide-derived macrolide antibiotics that target the ribosome. (A)** Structures of erythromycin, azithromycin, and telithromycin. **(B)** Exit tunnel for nascent polypeptide chains in the 50S bacterial ribosome. PT, peptidyl transferase active site where peptide bonds are formed. The blue dot marks the site of binding of macrolides and lincosamide antibiotic to 23S rRNA at the front of the tunnel. This binding blocks exit of the polypeptide chain (yellow) and halts translation elongation. Adapted from Walsh C. 2003. Antibiotics: actions, origins, resistance. ASM Press, Washington, DC.

bacteria, but are bactericidal for some Gram-positive bacteria. Macrolides, like tetracyclines, have also been used for livestock, primarily to prevent shipping sickness. There is some concern, as with the tetracyclines, that this nonclinical use is contributing to widespread bacterial resistance to this class of antibiotics.

Azithromycin (Figure 15-16) has been an exciting newer-generation macrolide. Azithromycin is one of the great triumphs of SAR to improve the antibiotic properties of an existing class of antibiotics. Not only is it an acid-stable antibiotic, allowing it to be taken orally, but it is also readily absorbed by the intestine. Because of its stability, long half-life (40 to 60 hours versus 1 to 2 hours for erythromycin), and excellent bioavailability in humans, azithromycin is highly effective in treating sexually transmitted bacterial diseases, such as chlamydial disease and gonorrhea, because it only needs to be administered orally in one or two closely spaced doses. This solves a problem that has plagued clinics that treat people with sexually transmitted diseases, in which a patient is given tetracycline or another antibiotic and instructed to take it daily for a week. If the patient abuses drugs or alcohol, or is homeless, the full regimen may not be taken. Also, the patient may not abstain from sexual contact until the antibiotic regimen has been completed. A patient who can be treated just once with azithromycin no longer needs supervised treatment at a clinic. Additionally, this limited regimen tends to ensure the patient will take the entire course of antibiotic, and thus be cured more quickly with less chance of transmitting the disease to others.

Unfortunately, azithromycin has one rather serious drawback. It is over four times as expensive as tetracycline antibiotics, which is an added problem for many developing countries where resistance to tetracycline, doxycycline, and other antibiotics commonly used for treating sexually transmitted diseases is beginning to become a serious problem. This illustrates yet another aspect that has only been partially addressed; namely, pharmaceutical companies need to make enough return from new antibiotics to pay for the expense of their development. This need is at odds with the needs of vulnerable impoverished populations for inexpensive antibiotics.

Lincosamides. **Lincosamides,** such as **lincomycin** and **clindamycin** (Figure 15-17), are synthesized by a condensation between a modified sugar and an amino acid derivative, but they have the same mechanism of action as the macrolides and bind the 23S rRNA near the same site. Perhaps the most widely used lincosamide is clindamycin. Clindamycin has been used extensively to treat infections caused by obligate anaerobic bacteria, such as *Bacteroides* species. Clindamycin is also one of the few antibacterial compounds effective against disease-causing protozoa such as *Giardia,* a cause of a persistent and sometimes life-threatening diarrhea. Clindamycin's strength—its effectiveness against obligate anaerobes—also proved to be its downfall; patients who took clindamycin experienced a decrease in the number of the obligate anaerobes that comprise the major populations of the colonic microbiota. In some of these patients, this disruption of the normal microbiota allowed the pathogen *C. difficile* to overgrow and produce powerful toxins that could kill the patient (see chapter 18).

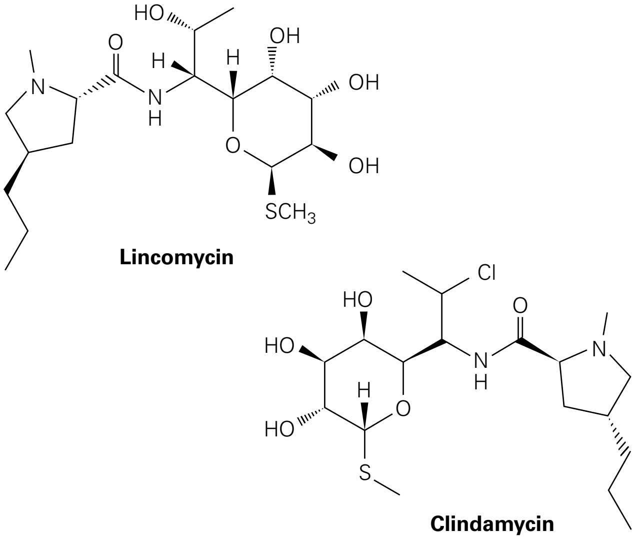


Figure 15-17. Structure of lincosamide antibiotics. Lincomycin is produced by *Streptomyces lincolnensis.* Clindamycin is chemically derived from lincomycin.

Antibiotics That Target DNA and RNA Synthesis

Antibiotics that inhibit bacterial replication generally do so by chemically modifying the DNA to generate double-strand breaks, by binding DNA and forming tight complexes that interfere with DNA replication, or by inhibiting enzymes associated with DNA replication (such as DNA gyrase) or DNA transcription (such as DNA-dependent RNA polymerase) (Figure 15-18). These antibiotics are effective only when they are selective for bacterial processes or enzymes.

Antibiotics that directly modify DNA (such as the DNA-cross-linking mitomycins) or that block bacterial replication by forming complexes with, or intercalating into, DNA and inducing double-strand breaks (such as bleomycins and actinomycins) are too toxic to use as antibiotics and are not considered further here.

Metronidazole and Nitrofurantoin. Although **metronidazole** and **nitrofurantoin** interfere with DNA synthesis, they do so not by inhibiting an enzyme, but rather by inducing double-strand breaks in the DNA (Figure 15-19). Metronidazole and nitrofurantoin, unlike other antibiotics, must first be activated by a bacterially encoded housekeeping

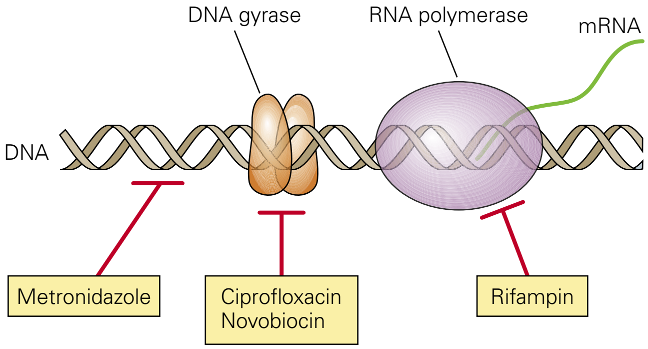


Figure 15-18. Antibiotics that inhibit DNA and RNA synthesis. Metronidazole works in anaerobic bacteria, where the nitro group is reduced by ferredoxins or flavodoxins into an active radical form that in turn causes double-strand breaks in the DNA and thiol adducts. Fluoroquinolones, such as ciprofloxacin and novobiocin, inhibit DNA gyrase. Rifampin (rifampicin) inhibits bacterial DNA-dependent RNA polymerase by blocking the formation of the phosphodiester bond in the RNA backbone, thereby preventing chain extension.

protein, either flavodoxin or ferredoxin, before it can attack DNA. Metronidazole has become important because it is one of the drugs used to eliminate *Helicobacter pylori*, which causes stomach ulcers, and because it is important for treating infections caused by anaerobic pathogens, such as *C. difficile*, that are resistant to most antibiotics. Because nitrofurantoin is concentrated in urine, where it can achieve more effective bactericidal levels than in other parts of the body, it is clinically used to treat UTIs.

The ferredoxins and flavodoxins found in microaerophiles and obligate anaerobes, but not aerobes, are capable of reducing the nitro groups of metronidazole and nitrofurantoin to a form that interacts with DNA and causes nicks in the DNA strands. The activated forms of these antibiotics could be considered to be mutagens. At one time, this feature of metronidazole raised questions about its safety, but because human cells do not covert metronidazole to a mutagen, these concerns were short-lived. Metronidazole has proved to be an antibiotic that has few side effects. Since such antibiotics are increasingly thin on the ground, metronidazole is looking better every day.

Metronidazole, like the lincosamide clindamycin, is not specific for bacteria. It is also effective against certain eukaryotic pathogens such as *Trichomonas vaginalis* (vaginitis) and *Giardia intestinalis* (diarrhea). These protozoa have the capability to activate metronidazole to its active, DNA-damaging form in common with anaerobic bacteria.

Rifampin (Rifampicin). A semisynthetic antibiotic produced by *Amycolatopsis rifamycinica*, rifampin (also known as rifampicin) (Figure 15-20) inhibits the activity of bacterial DNA-dependent RNA polymerase by binding to its β subunit and thereby preventing transcription into RNA. By analogy to the macrolides, rifampin binds to an exit tunnel in the DNA-dependent RNA polymerase, thereby

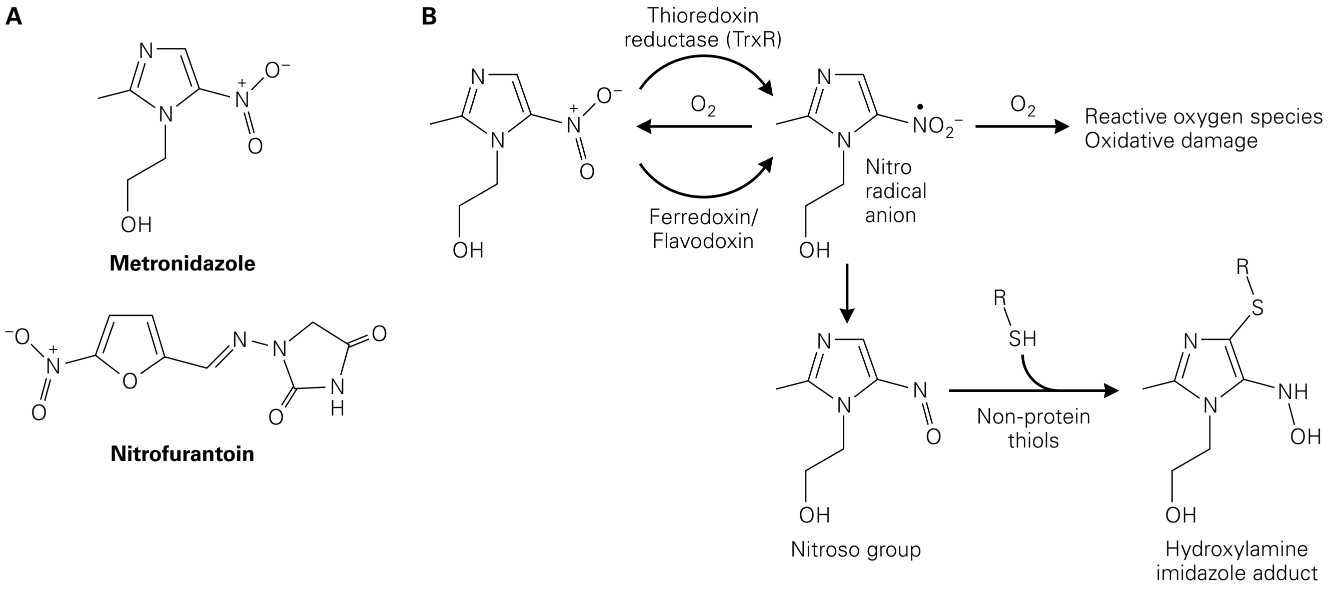
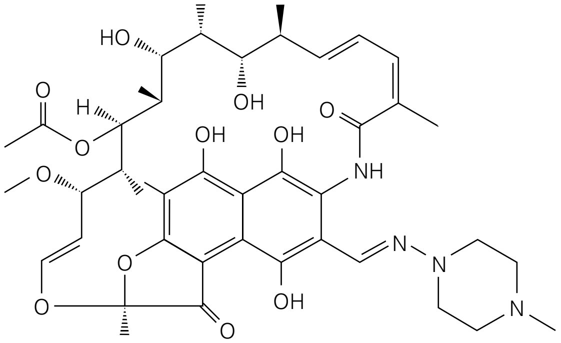


Figure 15-19. Structures and activation of metronidazole and nitrofurantoin. (A) Shown are the structures of the imidazole-containing antibiotics, metronidazole and nitrofurantoin. **(B)** Metronidazole must first be activated by reduction via flavodoxin or ferredoxin to an active radical form that can generate reactive oxygen species to cause double-strand breaks in the DNA or that can cause adduct formation with thiol groups. Reduction of metronidazole happens under anaerobic conditions, so it is most effective against anaerobic bacteria. Activation of nitrofurantoin is thought to occur by means of a similar mechanism, with the nitro group of the antibiotic being converted by flavodoxin into a nitronate anion radical in the bacterial cell.



Rifampin

Figure 15-20. Structure of rifampin (rifampicin).

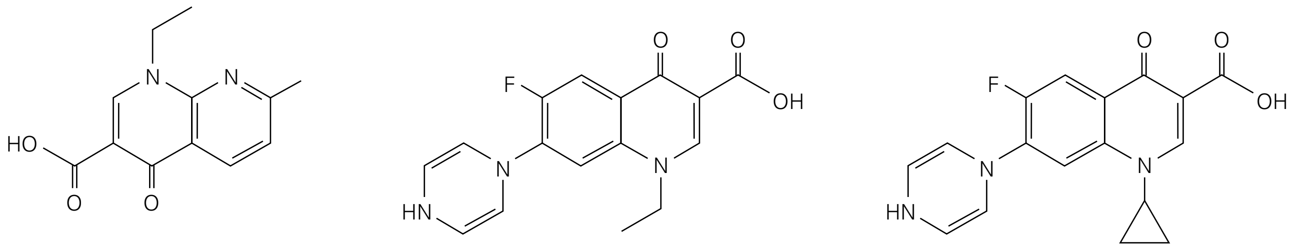
blocking the emergence of nascent RNA molecules. An important difference to keep in mind, however, is that the mouth of the tunnel in ribosomes blocked by macrolides is made of 23S RNA (see Figure 15-16B), whereas the tunnel in the RNA polymerase blocked by rifampin is made up of protein chains.

Although rifampin has always had its clinical uses (e.g., prophylactic use during outbreaks of meningitis caused by *Neisseria meningitidis*) and has been part of the drug combinations used to treat tuberculosis, it has only recently received much attention. The appearance of strains of *M. tuberculosis* that are resistant to isoniazid, one of the front-line antibiotics used to treat the disease, caused a renewed interest in rifampin. Unfortunately, resistance to rifampin can arise fairly easily as the result of single amino acid changes in the RNA polymerase, and so rifampin is most often used in combination with other antibiotics. Nonetheless, rifampin is one of the few drugs available for treating isoniazid-resistant tuberculosis. Rifampin is also used to treat other types of bacterial infections caused by strains that are resistant to the more commonly used antibiotics.

Quinolones and Fluoroquinolones. **Quinolones** are synthetic compounds that inhibit bacterial DNA synthesis and replication (Figure 15-18). For a long time, the only quinolone available, **nalidixic acid** (Figure 15-21), was very effective in treating UTIs caused by Gram-negative bacteria, but it was not particularly useful for many other types of infections. Then scientists added a fluorine group to the molecule and a new class of antibiotics useful for humans, the **fluoroquinolones**, was born. This new group of antibiotics caused considerable excitement in clinical circles because of its impressive antibacterial activity and good pharmacological properties.

Fluoroquinolones, such as **norfloxacin** and **ciprofloxacin** (Figure 15-21), are broad-spectrum bactericidal antibiotics that inhibit bacterial DNA replication by binding to and inhibiting the activity of DNA gyrase, which is a type II topoisomerase that introduces negative supercoils (i.e., relaxes positive supercoils) in DNA. During DNA replication, recombination, and transcription, DNA strands must be broken and reformed because of supercoiling. Fluoroquinolones block this essential process after the DNA gyrase has formed covalent links to and nicked each DNA strand, thereby creating fatal double-strand breaks in the bacterial chromosome. Fluoroquinolones enter cells via porins, and so can be used for treating intracellular bacteria, such as *Legionella* and *Mycoplasma*. Some of the newer generations of fluoroquinolones are more selective for the topoisomerase IV of Gram-positive bacteria, which unlinks (decatenates) chromosomes following replication.

Ciprofloxacin, commonly known as **Cipro**, gained considerable recognition during the anthrax attacks of 2001, when it was dispensed to a large population of people who had potentially been exposed to the anthrax spores through the U.S. mail system (more on this in chapter 20). At the time, Cipro was the only official FDA-approved antibiotic for the treatment of anthrax infection. Certain fluoroquinolones, such as Cipro, have poor activity against streptococci and anaerobes that comprise a majority of the resident microbiota of the mouth, colon, and vaginal tract. Thus, they are less likely than other antibiotics to disrupt the normal microbiota. Also, fluoroquinolones penetrate macrophages and polymorphonuclear leukocytes (PMNs) better than most other antibiotics and are thus useful for curing infections caused by bacteria that survive in phagocytes.



Nalidixic acid **Norfloxacin** **Ciprofloxacin**

Figure 15-21. Structure of fluoroquinolone antibiotics. Nalidixic acid was the first quinolone discovered. Norfloxacin and ciprofloxacin (also known as Cipro) are second-generation fluoroquinolones that gained considerable attention during the anthrax attacks of 2001.

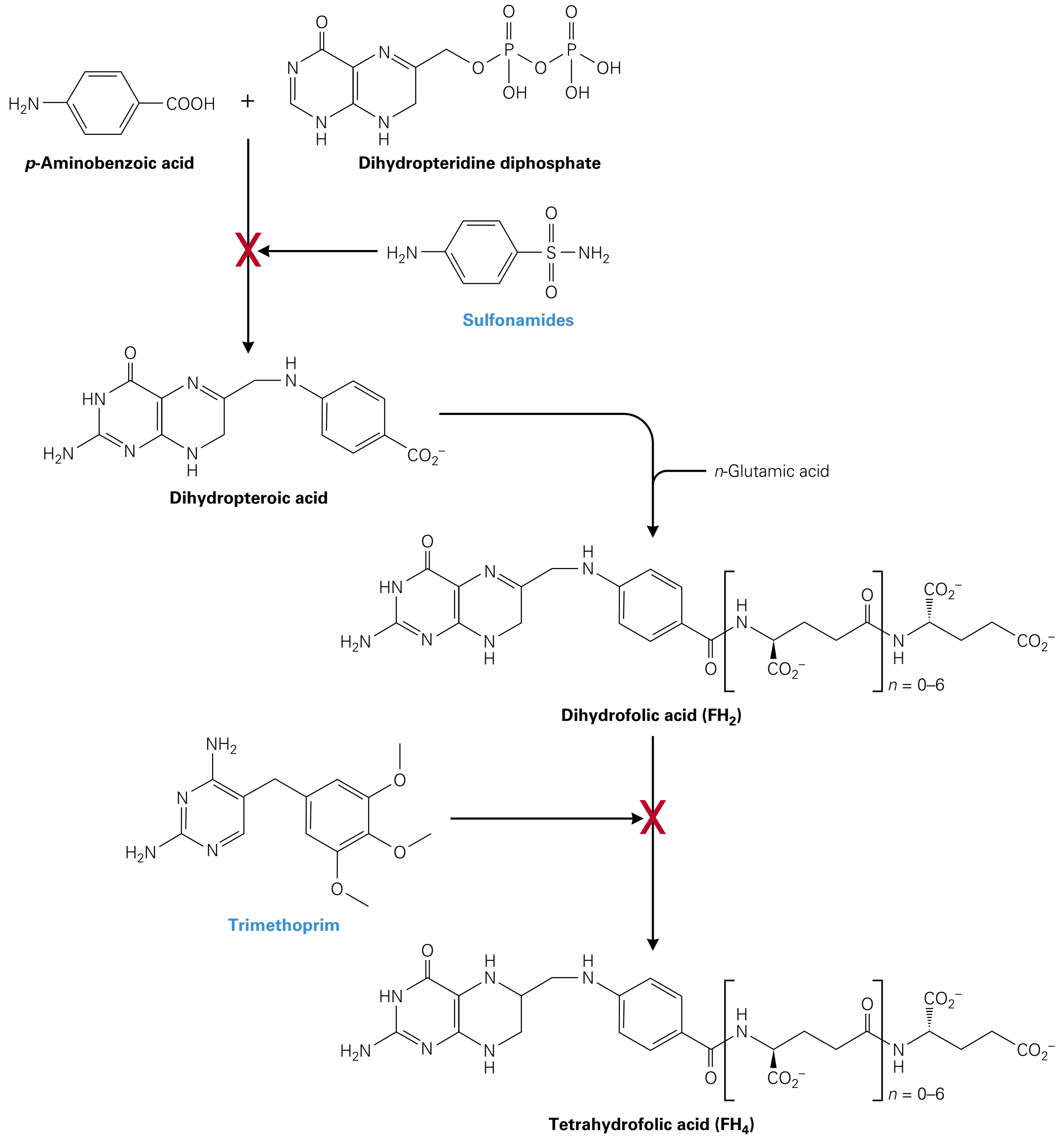

p-Aminobenzoic acid
Dihydropteridine diphosphate
Sulfonamides
Dihydropteroic acid
n-Glutamic acid
n = 0–6
Dihydrofolic acid (FH2)
Trimethoprim
Tetrahydrofolic acid (FH4)

Figure 15-22. Structure and action of trimethoprim and sulfonamides. Sulfonamides resemble the substrate *p*-aminobenzoic acid in structure and are thus able to competitively inhibit the action of dihydropteroate synthetase, the first enzyme in the tetrahydrofolate (FH_4) pathway. Trimethoprim resembles the substrate of dihydrofolate reductase, a later enzyme in the pathway, and competitively inhibits this step in synthesis.

The Achilles' heel of the fluoroquinolones is that a single mutation in DNA gyrase makes bacteria resistant to them. Overuse of fluoroquinolones in human medicine has had the predictable effect of giving rise to fluoroquinolone-resistant bacteria. Fluoroquinolones have even been approved for use in the water provided to chickens being raised in intensive chicken farming operations to prevent infections from sweeping through the bird population. The FDA's approval of this use of fluoroquinolones has been controversial. No one knows how much of a contribution this extensive new use of fluoroquinolones will make to resistance of bacteria such as *Salmonella* and *Campylobacter,* which are found in the avian intestine and can cause human disease. A strain of *Salmonella* Typhimurium (strain DT104) that has caused a number of outbreaks in recent years is resistant to fluoroquinolones and several other antibiotic classes.

Inhibitors of Tetrahydrofolate Biosynthesis

Tetrahydrofolic acid is an essential cofactor for 1-carbon transfer reactions that occur in pathways for synthesis of nucleic acids and *N*-formylmethionine (fMet). Mammalian cells require preformed folic acid because they do not make their own tetrahydrofolate (FH_4). Thus, inhibitors of the FH_4 pathway do not affect them. On the other hand, most bacteria make their own FH_4 *de novo* because they lack a transport system for its uptake. This makes the enzymes in bacteria that are involved in converting the precursors into FH_4 useful targets for antibiotics. Indeed, sulfonamide-containing drugs (also known as sulfa drugs) that inhibit a key step in the pathway were the first systemic antibiotics to become available in the 1930s and conceptually paved the way for the development of other antibiotics.

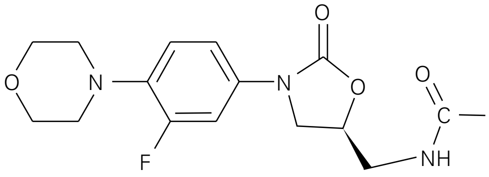
Trimethoprim and Sulfonamides. **Trimethoprim** and **sulfonamides** are inhibitors of enzymes in the bacterial pathway for the production of **tetrahydrofolic acid** (Figure 15-22). Sulfonamides are structurally similar to ***p*-aminobenzoic acid**, a substrate for the first enzyme in the pathway, and they competitively inhibit dihydropteroate synthase. Trimethoprim is structurally similar to dihydrofolic acid (FH_2), an intermediate in the pathway, and acts as a competitive inhibitor of dihydrofolate reductase, an enzyme that catalyzes the last step in the pathway. Trimethoprim and sulfonamides, such as sulfamethoxazole, are bacteriostatic and not usually used separately. However, a combination of the two drugs seems to act synergistically, resulting in bactericidal activity and decreased chances of resistance development, and is effective against bacterial and fungal respiratory infections.

The Newest Antibiotics

There have been a few new antibiotics introduced in recent years, two of which are members of known classes of antibiotics. As mentioned earlier, tigecycline is a tetracycline derivative with an added glycyl-glycyl group that helps overcome the resistance to tetracycline (Figure 15-15). The **ketolides** are new derivatives of macrolide antibiotics, in which the cladinose sugar group of erythromycin is substituted with a keto group and a cyclic carbamate group is attached to the lactone ring. These modifications provide ketolides with a much broader spectrum than other macrolides. Because they bind to two sites in bacterial 23S rRNA, ketolides are effective against macrolide-resistant bacteria. The only ketolide currently on the market is **telithromycin** (Figure 15-16). However, serious potential side effects, including liver failure, have limited the use of this antibiotic.

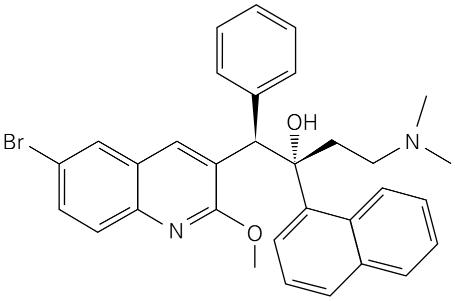
A completely new class of antibiotics, indeed the first new class in decades, has been given the tongue-twisting name **oxazolidinones**. Zyvox, the name under which the first member of this class, **linezolid** (Figure 15-23), is being sold, is easier to pronounce. The oxazolidinones are completely synthetic and bind the large ribosomal subunit to the location at which the macrolides, lincosamides, and streptogramins bind (Figure 15-13). However, the oxazolidinones seem to act at an earlier step in translation than these other antibiotics. They seem to bind to the peptidyl transferase site and block formation of the first peptide bond during protein translation. Linezolid is primarily used to treat infections by Gram-positive bacteria, such as VRE, that are resistant to other antibiotics. The oxazolidinones have high oral bioavailability and few side effects. Unfortunately, for a new drug introduced in 2000, resistance has already started to appear due to base changes in the 23S rRNA.

Among the more promising new IND antibiotics reported recently are **diarylquinoline** compounds (Figure 15-24) that specifically inhibit *Mycobacteria*, including drug-resistant *M. tuberculosis*. The lead compound in this



Linezolid

Figure 15-23. Structure of the oxazolidinones. The first member of this class of synthetic antibiotics is linezolid (Zyvox).



Bedaquiline

Figure 15-24. Structure of bedaquiline. This diarylquinoline compound blocks the growth of *Mycobacteria* by inhibiting the proton pump for ATP synthase. Note that this mechanism of action is unlike other quinolone antibiotics, which target DNA gyrase.

group, **bedaquiline**, was identified by medium throughput whole-cell screening for inhibition of bacterial growth. Bedaquiline only inhibited the growth of *Mycobacteria* and did not inhibit other bacterial species. This compound fits Lipinski's rules for a good drug candidate and showed excellent pharmacokinetic and pharmacodynamic profiles in animal models.

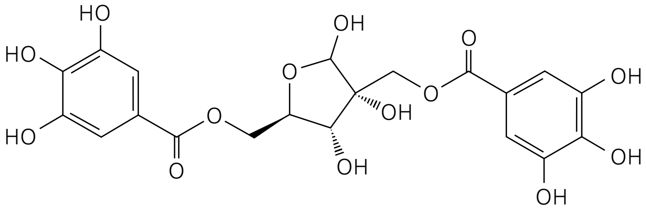
Resistance studies indicated that bedaquiline inhibits a unique target: the essential membrane-bound mycobacterial ATP synthase that synthesizes ATP. All bacteria contain a homologous ATP synthase, and it was unprecedented that these compounds specifically inhibited the enzyme in *Mycobacteria*. Efficacy studies in animal models of tuberculosis suggested that diarylquinoline was effective alone and in combination with other drugs in combating infection. This promising new kind of antibiotic emerged successfully from human trials and in 2012 received approval by the FDA. Bedaquiline was the first new drug to be added to the arsenal of antituberculosis drugs in more than 40 years. The fact that resistance can develop by simple point mutations in genes encoding the ATP synthase means that diarylquinolines will need to be used judiciously and only in combination with other antituberculosis drugs.

The Newest Antibiotic Targets

Some recent antibiotic discovery strategies have moved away from targeting essential bacterial housekeeping functions. Instead, various pathways involved in virulence are being targeted for inhibition, such as quorum sensing, toxin delivery, virulence regulation, and bacterial adhesion. **Quorum sensing** provides regulatory control over specific groups of genes, including those encoding biofilm formation and various virulence factors such as toxins, secretion systems, and adhesins. Inhibition of these bacterial cell-cell communication systems may result in attenuation of virulence (bacteriostatic rather than bactericidal) and may thus represent attractive new targets for antibiotic discovery.

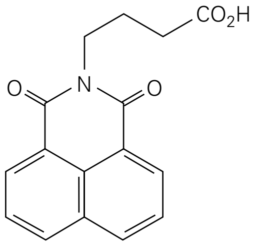
Recent studies suggest that targeting quorum sensing may be particularly relevant for bacteria that use quorum sensing to regulate the expression of virulence genes and that are becoming increasingly resistant to other antibiotics, such as MRSA and *Pseudomonas aeruginosa*. Indeed, a recent structure-based screen of a small molecule library that targeted the quorum-sensing regulator of MRSA turned up a small molecule inhibitor that occurs as a natural product in the bark of witch hazel, called **hamamelitannin** (Figure 15-25). This compound also controlled MRSA infection by affecting peptidoglycan biosynthesis and thereby increasing antibiotic susceptibility of biofilms toward vancomycin and other antibiotics.

A very exciting recent finding is that azithromycin (Figure 15-16) exhibits strong quorum-sensing antagonistic activity against virulence factor production, biofilm formation, and oxidative stress responses in *P. aeruginosa*. Even though *P. aeruginosa* infections, especially in chronic infections of the lung in cystic fibrosis patients, are normally quite refractory to killing by macrolides, azithromycin has been reported to positively influence the clinical outcome in patients with lung infections.



Hamamelitannin

Figure 15-25. Structure of hamamelitannin. This natural product, isolated from extracts of the medicinal plant witch hazel, blocks quorum sensing in methicillin-resistant staphylococci.



Virstatin

Figure 15-26. Structure of virstatin. This small molecule inhibitor blocks dimerization and activation of ToxT, a transcriptional regulator of the *Vibrio cholerae* virulence genes *ctx* and *tcp*, encoding cholera toxin and toxin-coregulated pilus (TCP), respectively.

Another success story involved high-throughput phenotypic screening of compound libraries for direct inhibition of virulence, which resulted in discovery of a small molecule **virstatin** (Figure 15-26) that inhibited dimerization and activation of ToxT, a known transcriptional regulator of cholera toxin and toxin-coregulated pilus production in *V. cholerae*. Virstatin blocked toxin production and protected mice from intestinal colonization with the bacteria.

One should keep in mind that these new classes of targets, while interesting, may result in inhibitors that are largely bacteriostatic and that have very narrow activity spectra. Bacteriostatic antibiotics are less desirable in patients with compromised immune systems. A narrow activity spectrum means that physicians need to be extremely certain that a condition is solely caused by the bacterium that will respond to this drug, and this is not always possible. Last, FDA approval depends on showing that these new drugs are at least as efficacious and safe as potent drugs that are already approved.

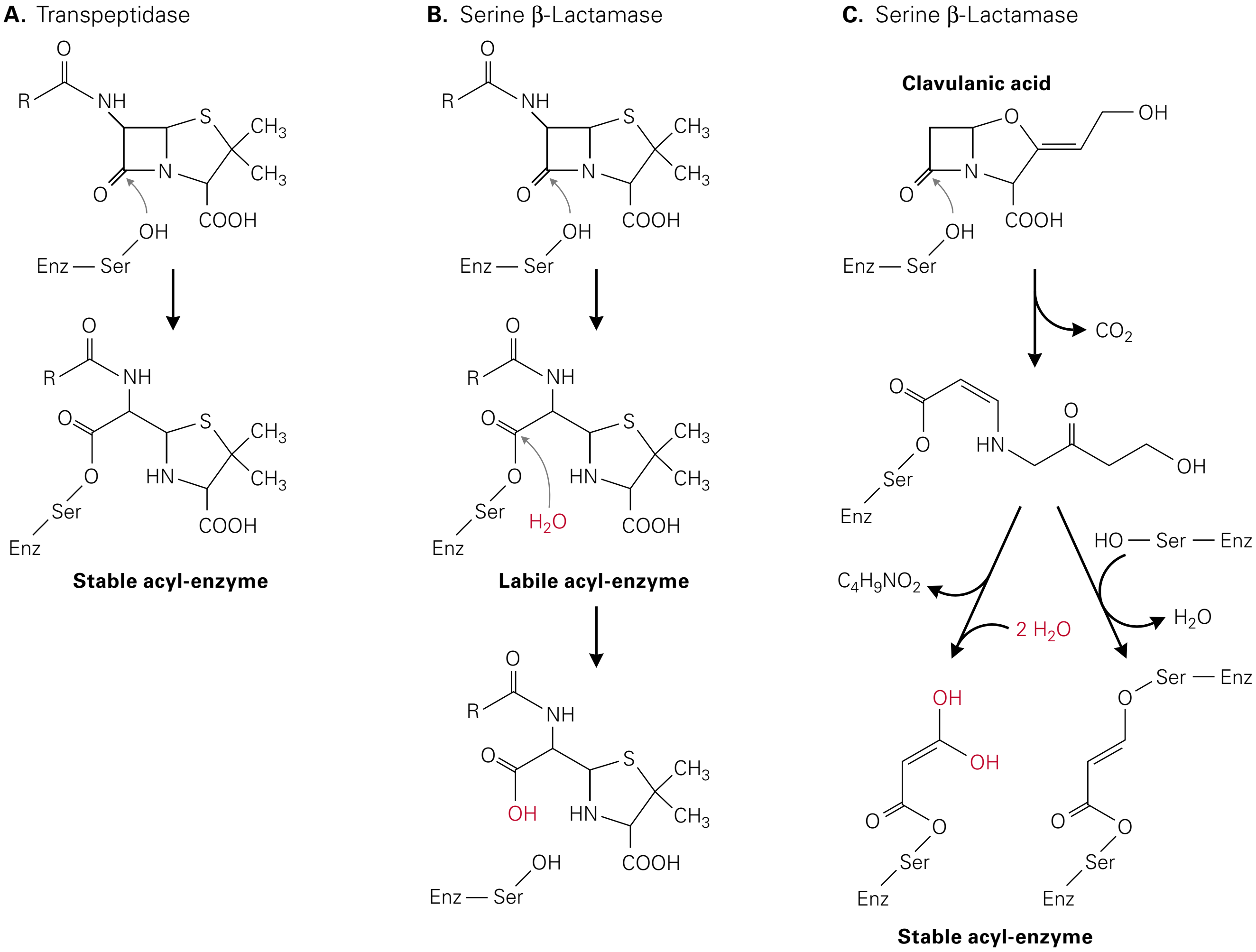
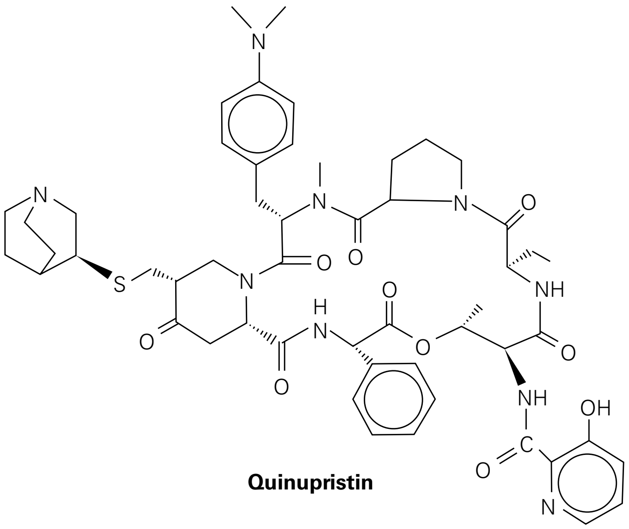


Figure 15-27. β-lactam-derived augmenters to enhance β-lactam-antibiotic efficacy. β-lactam augmenters are themselves poor antibiotics, but are often combined with the active β-lactam antibiotics to prevent their degradation by β-lactamases. **(A)** β-lactams act as substrate-based inhibitors of cell wall transpeptidases by forming stable acyl-enzyme adducts with serine at the active site. **(B)** β-lactamases inactivate β-lactam antibiotics by cleaving the β-lactam ring of the β-lactam to form an acyl-enzyme intermediate that is subsequently hydrolyzed by water to release the inactivated β-lactam. **(C)** Clavulanic acid and other related β-lactam-derived compounds are mechanism-based inactivators (also known as suicide-substrate inhibitors) of β-lactamases. The resulting acyl-enzyme intermediate that is formed has a reactive α,β-unsaturated carbonyl that is subject to attack by another active site nucleophile to form a more stable acyl-enzyme adduct.

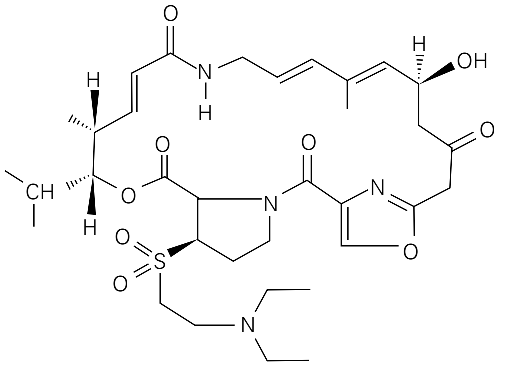
Strategies for Enhancing Antibiotic Efficacy

There has been considerable success in screening for and using drug design approaches to find **augmenters** (i.e., helpers) of existing antibiotics, which make existing antibiotics more potent or stable. One of the most successful examples of this approach is **clavulanic acid** (Figure 15-27), an irreversible mechanism-based (suicide substrate) inhibitor of one class of β-lactamases (the products of antibiotic resistance genes). Clavulanic acid was initially discovered as a natural product with poor antibiotic activity itself, but it strongly inhibits one class of β-lactamases with a catalytic serine at the active site. Synthetic clavulanic acid is often included in drug formulations with other β-lactam antibiotics to prevent their degradation by β-lactamases. Clavulanic acid combined with the penicillin derivative amoxicillin is marketed as the drug **Augmentin.** Unfortunately, resistance caused by a second kind of metal-dependent β-lactamase that is not inhibited by clavulanic acid emerged, and so another augmenter, called colastatin, had to be found that inhibits this second class of β-lactamases.

Screening and combinatorial methods have also been applied to combating vancomycin resistance. Vancomycin interferes with bacterial cell wall biosynthesis by binding to the D-Ala-D-Ala linkage of peptidoglycan precursors. Bacteria become resistant to vancomycin by modifying this D-Ala-D-Ala linkage. Resistance genes encode enzymes that



Quinupristin



Dalfopristin

Figure 15-28. Structure of the two streptogramins in Synercid. Quinupristin and dalfopristin are protein synthesis inhibitors that act synergistically by cooperatively binding to the 50S ribosomal subunit.

substitute D-Ala-D-Ala with D-Ala-D-lactate in the cell wall precursors. The presence of D-Ala-D-lactate in the cell wall and its precursors reduces the affinity of vancomycin for the peptidoglycan. In this case, library screening included compounds that are not themselves antibiotics, but were able to resensitize the resistant bacteria to the antibiotic. One screen identified small compounds that selectively cleaved the modified peptidoglycan D-Ala-D-lactate linkage. Because these D-Ala-D-lactate linkages were removed, the bacteria once again became sensitive to vancomycin.

Streptogramins are usually overlooked in most textbooks because they have not been used to treat human infections until very recently. The appearance of **Synercid** on the market changed that picture in a radical way. Synercid is a combination of two streptogramins, **dalfopristin** and **quinupristin** (Figure 15-28). The reason the mixture is needed is that each of the drugs alone is bacteriostatic, but together they are bactericidal. Synercid has the same mechanism of action as the macrolides and lincosamides; it binds to the same portion of the 23S rRNA in the 50S subunit (Figure 15-13). Dalfopristin binding blocks the peptide exit tunnel and enhances the binding of quinupristin to a nearby site by a hundredfold, which prevents elongation of the peptide chain and causes release of incomplete polypeptides.

Synercid was developed in response to the growing resistance of *Enterococcus* species and *S. aureus* to vancomycin, the last-ditch treatment of infections caused by these two Gram-positive opportunists. Streptogramins would seem to be an ideal solution to the question of how to develop a new antibiotic that would be effective against the multiresistant bacterial threats looming in our future. Since streptogramins have not been used to treat human infections in the past, resistance would not be a problem—at least not at first, right? Well, not quite. It turns out that streptogramins have been used widely in agriculture for a number of years, under the names virginiamycin and pristinamycin, respectively, to treat and prevent disease in animals being raised under crowded conditions. Not surprisingly, resistance to streptogramins in nature has emerged, and the CDC is now monitoring the emergence of streptogramin-resistant strains. The question remains of how separated the consequences of agricultural use of antibiotics actually are from the consequences of human use of antibiotics. Another complicating factor, which is covered in greater detail in the next chapter, is that because the macrolides, lincosamides, and streptogramins bind to overlapping sites on the bacterial ribosome, some of the mechanisms bacteria develop to resist the action of these antibiotics confer resistance to all three classes. This means that use of one class can select for resistance to the other classes in this triumvirate.

The Continuing Challenge

The few new antibiotics that are trickling through the pipelines present a quandary for physicians, pharmaceutical companies, and public health institutions. There is understandable pressure to reserve these new drugs for the cases in which nothing else works, yet it is difficult to ask physicians, who have to make accurate guesses as to what antibiotic to use on their very sick patients, to forgo the latest antibiotics, especially if multidrug-resistant bacteria have become a problem in their hospitals. It is also difficult to ask pharmaceutical companies to restrict the sales of an antibiotic that has taken hundreds of millions of dollars, and decades, to develop and test. Fear of not being able to recoup research and development costs and make a sizable profit has been one of the arguments used by pharmaceutical companies to justify their retreat from antibiotic research. Clearly, the development and judicious use of new antibiotic drugs is an ethical and economic challenge.

There is also the challenge of how to control the use of the older antibiotics that still work against many bacterial infections. Physicians have been accustomed to making their own decisions as to what antibiotic to prescribe, and they are already chafing at the restrictions on medical practice being imposed by HMOs. There are prudent use guidelines that have the imprimatur of the American Medical Association, but they are often ignored. Two of the most widely flouted guidelines are the one that discourages the use of antibiotics to treat sore throats and flu, and the one that discourages the use of antibiotics to treat ear infections in children until they have persisted for 24 to 48 hours. Physicians like to please their patients and have an economic as well as psychological incentive for doing so. What does a physician do when a patient threatens to go elsewhere? Also, complaints lodged by patients against physicians can get the physician in trouble with hospital administrators. Clearly the problem of enforcing prudent antibiotic use goes beyond scientific considerations and spills over into economic and political areas. Perhaps the future will see the solution to these problems materialize and a new era of prudent use of antibiotics, in which they are revered and conserved as the vital resource they are, will be ushered in.

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Questions

1. How do antiseptics, disinfectants, and antibiotics differ from one another?

2. Why might it be harder for a bacterium to become resistant to a disinfectant than to an antibiotic?

3. Many of the antibiotics described in this chapter have a similar type of action: they bind to an important bacterial target and inhibit its activity. Are there exceptions to this rule?

4. The pharmaceutical industry has been trying to develop antibiotics by solving the crystal structure of a protein target and designing molecules that fit into the active site of this target (rational drug design). So far, this strategy has not been nearly as successful as expected. Why did scientists think this would be superior to the old ways of finding antibiotics and what are some of the reasons this approach might fail?

5. Many of the aminoglycoside antibiotics are much less effective against *Escherichia coli* under anaerobic conditions than under aerobic conditions. Assume that the rates of protein synthesis are about the same in both cases and that the antibiotic inhibits protein synthesis *in vitro*. How would you explain the reduced effectiveness under anaerobic conditions?

6. Although *in vitro* tests of susceptibility to antibiotics are widely used and considered useful, there have been cases in which the *in vitro* tests have not predicted how well the drug would work when administered to humans. This works both ways. Some antibiotics that are effective *in vitro* fail when administered to humans, and some antibiotics that test resistant *in vitro* actually work when used therapeutically. Give some explanations for both of these outcomes.

7. In the quest for new antibiotics, what are the pharmaceutical companies trying to do? What criteria would you use to judge whether to develop a new antibiotic? What is the attraction of sticking with known antibiotics and trying to modify their structures, rather than looking for new antibiotic targets?

8. For the following antibiotics, briefly describe how each kills or inhibits the growth of bacteria: β-lactams, glycopeptides, aminoglycosides, tetracyclines, quinolones, and macrolides.

9. An ideal antibiotic must have certain characteristics in order to be effective in human infections. Name four characteristics of a good antibiotic.

10. Indicate whether the following statements are TRUE or FALSE:

______ Aminoglycosides are bacteriostatic antibiotics.

______ Quinolones affect the replication of DNA.

______ Pharmaceutical companies synthesize antibiotics in the laboratory.

______ Antibiotics are only administered for short periods of time because they have side effects on the patient.

______ Broad-spectrum antibiotics are often used when the pathogen has not been identified.

______ Broad-spectrum antibiotics mainly kill Gram-positive bacteria.

______ Tetracyclines cause discoloration of teeth in children.

______ Rifampin inhibits the activity of bacterial RNA polymerase.

______ Streptogramins inhibit bacterial DNA replication.

______ Trimethoprim inhibits tetrahydrofolate biosynthesis.

______ Ketolides are new derivatives of erythromycin antibiotics.

______ The β-lactam ring in penicillin can be cleaved to inactivate the antibiotic.

______ The β-lactam ring in penicillin can be modified to increase the spectrum of antibiotic activity.

______ Taking antibiotics can make you more susceptible to other infections.

______ Isoniazid, one of the primary antibiotics used against tuberculosis, inhibits mycolic acid biosynthesis.

______ Cephalosporin acts primarily on bacteria by inhibiting the transpeptidation reaction of peptidoglycan biosynthesis.

______ Tetracycline is a broad-spectrum antibiotic that acts on both Gram-negative and Gram-positive bacteria by binding the 30S ribosomal subunit and inhibiting protein synthesis.

______ Metronidazole is a broad-spectrum antimicrobial that acts by forming reactive nitro species that inhibit DNA replication in anaerobic bacteria.

Solving Problems in Bacterial Pathogenesis

1. A group of researchers at the university veterinary diagnostics laboratory isolated a new bacterium from the blood and stool of several horses that became ill and died at a local farm. Prior to death, symptoms included disorientation, loss of motor function, and flaccid paralysis, so the researchers suspected central nervous system (CNS) involvement and possible production of neurotoxin. Based on 16S rRNA comparison, they found that the new bacterium is related to the Gram-positive bacterium *Clostridium botulinum,* and they subsequently named it *Clostridium equiniae*. They also determined that the bacterium is sensitive to metronidazole, but resistant to β-lactam antibiotics, such as penicillin, or to macrolide antibiotics, such as erythromycin and azithromycin. However, although *C. equiniae* is resistant to azithromycin, the researchers found that when they treated infected mice with metronidazole in combination with azithromycin, the LD_{50} value went from 10 for control without antibiotic treatment to 10^4 for treatment with metronidazole alone to 10^7 for combined treatment with azithromycin, and the mice recovered from paralysis much sooner with the combined treatment than with metronidazole treatment alone. Interestingly, when they were plating out the bacteria to determine the LD_{50} values, the researchers observed that the colonies isolated from the control mice and mice treated with metronidazole alone were mucoid, but those from mice treated with both antibiotics were not.
    a. What possible mechanism(s) could account for all of these observations (i.e., change in LD_{50} value, faster recovery from paralysis, and change in mucoid phenotype)? Be sure to provide your rationale.
    b. Provide an experiment that could be performed to confirm your hypothesis.

2. You are a member of a research team working for the CDC that is investigating a recent outbreak of food poisoning due to contaminated sandwiches at a catered event. During the investigation, your team identified two bacteria that contributed to the food poisoning, apparently due to contamination from one of the sandwich makers who handled the food without first washing hands. Of the 100 guests who attended the event, 78 experienced self-limiting symptoms of nausea, stomach cramps, and diarrhea that started as little as 30 minutes after eating the food and lasted for one to two days. For 12 of these individuals symptoms were more severe, with both vomiting and bloody diarrhea, which required hospitalization for intravenous rehydration and treatment with the antibiotics erythromycin, ciprofloxacin, and Augmentin. Diagnostic evaluation of bacterial cultures from blood, vomit, and fecal samples identified the presence of two bacteria known to be associated with food poisoning. The Gram-positive *Staphylococcus aureus* was found in the vomit and fecal samples, while the Gram-negative *Campylobacter jejuni* was found in all three of the samples. All of the 12 patients improved over the course of two to three days and were discharged. Through your studies, you find that both bacteria are susceptible to erythromycin and ciprofloxacin. While both strains are resistant to most β-lactams, the *S. aureus* strain can be effectively treated with Augmentin. However, the *C. jejuni* strain is still resistant.
    a. Provide an explanation for the observed antibiotic profile. Be sure to include how each antibiotic works as well as the most likely mechanism for each of the observed resistances and sensitivities to Augmentin.
    b. What is a potential problem with using antibiotics to treat the systemic infection? Explain why the chosen antibiotic treatment regimen did not cause this adverse outcome.

16

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CHAPTER 16

Antibiotic Resistance

Resistance to antibiotics appeared in clinical isolates shortly after antibiotics were introduced onto the battlefield in our war against bacterial diseases. At first, research on bacterial resistance to antibiotics focused primarily on basic science issues, such as the mechanism of resistance and regulation of resistance gene expression. Now, however, we are beginning to appreciate the economic consequences of increasing bacterial resistance. Hospital administrators and government regulators who have to confront health care costs have made a troubling discovery: resistance makes it not only harder to cure disease, but also more expensive! Lawsuits are now being brought against hospitals where patients have acquired drug-resistant nosocomial infections. Concern about resistant bacteria has also moved from the hospital to the farm; antibiotic-resistant bacteria have become the center of the controversy over the use of antibiotics in agriculture. To make matters worse, whereas most isolates were initially resistant only to a single class of antibiotics, we are facing an even direr problem of multidrug resistance. Clearly, concern about antibiotic resistance has come out of the ivory tower and into the real world.

The Dawning of Awareness—Uh, We Have a Problem!

The 1990s was the decade when the public first began to take real notice of the growing problem of bacteria that were increasingly becoming resistant to many of the mainline drugs used to treat infections with those bacteria (Table 16-1). Prior to this, physicians tended to downplay the importance of antibiotic-resistant strains of disease-causing bacteria, because in virtually all cases there were still antibiotics—sometimes many antibiotics—that continued to work and could be used instead. However, a shift in attitude had already begun to appear. Whereas having to turn to a second antibiotic was once considered a treatment failure, gradually the definition of treatment failure became failure to find a successful antibiotic after trying several. As long as the physician finally hit upon an effective antibiotic, the treatment was considered a success. Patients did not always agree; certainly those with serious systemic infections sometimes suffered irreversible damage to important organs or suffered strokes because the infection was not brought under control quickly enough.

Arguably, the first groups to become publicly concerned about antibiotic-resistant bacteria were the officials of health insurance companies and HMOs. Infections caused by resistant bacteria were proving to be expensive (Box 16-1),

Table 16-1. Evolution of antibiotic resistance

ANTIBIOTIC	YEAR DEPLOYED	YEAR RESISTANCE OBSERVED
Sulfonamides	1930s	1940s
Penicillin	1943	1946
Streptomycin	1943	1959
Chloramphenicol	1947	1959
Tetracycline	1948	1953
Erythromycin	1952	1988
Vancomycin	1956	1988
Methicillin	1960	1961
Ampicillin	1961	1973
Cephalosporins	1960s	1960s
Ciprofloxacin	1987	1990
Linezolid	1999	2003
Daptomycin	2003	2005

which is even more so the case today. Resistant bacteria were also costing state governments money. It cost New York City nearly a billion dollars to bring a drug-resistant tuberculosis outbreak in the mid-1990s under control. About this same time, the New York Chamber of Commerce approached the state legislators to ask what was being done about antibiotic resistance. Even in the absence of a high-profile epidemic, businesses were losing money because of days taken off work and higher health care costs, and there appeared to be no end to this problem. Congress held hearings about antibiotic-resistant bacteria and their potential impact on human health.

Predictably, the media fell on the issue with their usual gusto. Headlines such as “The End of Antibiotics” and “Return to the Pre-Antibiotic Era” began to appear in news outlets and magazines. Not surprisingly, the content of most of these articles was sensational and frightening. Even the normally staid professional scientific journals displayed covers and headlines about the antibiotic resistance problem that made the mainstream media seem conservative by comparison. The media soon discovered the agricultural use of antibiotics, and the possible detrimental impact of this use on human health began to be the subject of additional news articles and TV programs. Environmental advocacy groups began to consider antibiotic use and antibiotic resistance an issue on which they needed to take a stand. Even the Humane Society got involved because it realized that severely restricting the use of antibiotics in agriculture might force improved hygiene and reduced crowding, changes that would improve the quality of life for the animals being raised.

Pharmaceutical companies responded to the steady increase in resistance by performing a highly effective “me-too, me-better” strategy to improve existing antibiotics, especially the β-lactam and macrolide classes. Several generations of new β-lactam antibiotics appeared over the ensuing years that stayed one step ahead of resistance for a time. As a result, several new classes of antibiotics appeared. However, eventually the chemical options to modify known antibiotics in order to outmaneuver the growing resistance problem became limited around the same time that the discovery of new classes of antibiotics nearly ceased (see Figure 15-5). These setbacks, along with drastic changes in business models, contributed to the decisions of many pharmaceutical companies to curtail antibiotic discovery and development, which has only exacerbated the resistance problem.

Regrettably, a daunting number of important questions remained unanswered about the mechanisms of resistance and transmission. When Gram-positive bacteria began to reassume prominence as leading causes of human infections, it became evident that very little was known about their mechanisms of resistance or the spread of resistance genes, such as plasmids and transposons carrying antibiotic-resistance genes. Moreover, many of the questions that began to dominate the debate over patterns of antibiotic use and possible preventive strategies in human medicine or agriculture turned out to be questions about the ecology and evolution of antibiotic-resistant bacteria and their genes. This area had been almost untouched even in the heyday of research on antibiotic-resistant bacteria. With the dawning realization that horizontal gene transfer (HGT) is highly prevalent among environmental and pathogenic bacteria, discovered through comparative sequence analysis of the large repertoire of bacterial genomes, the need to understand antibiotic resistance mechanisms is at a critically urgent state. Luckily, the information gleaned from these genomes is also providing new and exciting alternative strategies for combating infections with antibiotic-resistant bacteria.

How Did We Get to Where We Are?

One of the major contributors to the development of resistance is the tremendous genetic plasticity of bacteria. As discussed later in this chapter and elsewhere in this book (chapter 7), bacteria have many mechanisms by which they can acquire mutations and exchange genetic material. Bacteria exist in phenomenally high populations within environmental and host niches and as members of complicated microbial communities, including mixed biofilms. In this background, resistance can quickly emerge in response to strong selective pressure by exposure to antibiotics, especially against bacteriostatic antibiotics, and resistance mechanisms, once they arise, can then spread rapidly among bacterial species. About 50% of antibiotic

Box 16-1.

The Co$t of Antibiotic Resistance—A Telling Example

During the 1990s, *Staphylococcus aureus* became one of the most common causes of hospital-acquired infections in the United States and other developed countries. Hospital-acquired infections, especially postsurgical infections, are much more common than they should be. According to the CDC, in the United States alone about 2 million hospital patients will acquire such an infection and at least 23,000 people will die each year as a direct result of infections with MDR bacteria. *S. aureus* has become more and more resistant to a variety of antibiotics, with the methicillin-resistant *S. aureus* (MRSA) strains being the ones that are currently the most troublesome. Actually, the acronym "MRSA" would be more accurately rendered as multidrug-resistant *S. aureus* because these strains are usually resistant to several antibiotics other than methicillin. The only drug currently able to effectively control MRSA infections is vancomycin, and isolated reports of MRSA strains with reduced susceptibility to vancomycin have begun to appear in the United States.

In a study estimating the costs of MRSA infections in New York City in 1995, about 21% of all *S. aureus* infections acquired in hospitals or in the community were caused by MRSA strains. In the case of community-acquired infections, the additional cost per patient to treat these infections was about $2,500. Frequently, these patients had to be hospitalized. For hospital-acquired infections, the additional cost was higher, $3,700 per patient, probably because the patients involved were sicker than the patients with community-acquired infections and were thus less able to fight the disease.

In a 2009 study estimating the impact of antibiotic-resistant infections within a large urban public teaching hospital in Chicago, drug resistance was attributed to 6.5% mortality, double that without such infections. The length of stay was increased by an average of 11 days at an additional treatment cost of $18,600 to $21,200 and a combined hospital and societal (loss of productivity) cost of about $71,000 per episode, with a total cost for the 188 patients with MDR infections in that hospital for that year amounting to a staggering $13.35 million.

The higher cost of treating MRSA infections was due to a variety of factors. First, vancomycin is more expensive than the drugs normally used to treat *S. aureus* infections. Second, it is often necessary to isolate patients to keep them from infecting other patients. Third, patients with MRSA infection stayed longer in the hospital. The increased financial cost was not the only toll taken by MRSA. The death rate for patients with MRSA infections was higher than the death rate due to infections caused by susceptible *S. aureus* strains. The resistant strains are not necessarily more virulent than the susceptible ones, but their resistance makes it harder to control them with antibiotics. Societal costs also included economic costs to the patient. Longer hospital stays mean more days lost at work and more disruption of family life. Patients who died obviously paid the highest cost, but even patients who survived could leave the hospital with irreversible damage to vital organs, such as the brain, lungs, and kidneys. Keep in mind that MRSA strains in the United States are largely still treatable with vancomycin, but there have been over a dozen cases of vancomycin-resistant MRSA strains reported over the last decade and the looming fear is that they will increase.

Sources:

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use in the United States has been estimated to be inappropriate, in that antibiotics are often overprescribed, prescribed at the wrong dosages or durations for bacterial infections to be completely cleared, or prescribed unnecessarily for viral infections.

One of the greatest sources of resistance continues to be the use of antibiotics to enhance the growth of livestock in crowded feedlots. At this writing, the United States Congress has yet to pass the Preservation of Antibiotics for Medical Treatment Act (PAMTA), which would greatly limit the use of antibiotics as food animal growth supplements. This important legislature remains stalled in the House Energy and Commerce Committee's Subcommittee on Health. Crowding, homelessness, poor nutrition and sanitation, and inadequate routine medical care remain problems that promote the spread of antibiotic resistance in developed as well as developing countries. In developed countries, certain social organizations, daycare centers, schools, and hospitals remain sources of bacteria harboring antibiotic-resistance genes that can spread to visitors and family members. International travel has become commonplace, so resistant bacteria can rapidly be spread worldwide.

Another source of antibiotic resistance is long-term treatment with antibiotics as a prophylactic measure in cases of immunosuppression due to greater life expectancy, diseases such as HIV infection, invasive medical procedures such as organ transplants, and the use of implanted medical devices that support biofilm growth. At the same time, public health infrastructures have eroded in the United States and other developed countries, which set up conditions for the rapid spread of resistance. These formidable contributing factors are beginning to be addressed, especially with the increasing appearance of community- and hospital (**nosocomial**)-acquired bacterial infections caused by drug-resistant bacteria.

And Now the Really Scary Part. . .

Emergence and Challenge of Multidrug Resistance (MDR)

Nowhere has the impact of drug resistance in the fight against the spread of infectious diseases become grimmer than in the hospital setting, where antibiotic interventions are essential for successful clinical outcomes, especially within that vulnerable population. Yet nosocomial infections from drug-resistant bacteria are escalating at an alarming rate, as described in the 2013 Centers for Disease Control and Prevention's (CDC) report on *Antibiotic Resistance Threats in the United States* (https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf). To make matters worse, by the late 1990s it was becoming increasingly difficult to find drug treatment regimens that would quickly clear infections due to the rise of **multidrug-resistant (MDR)** strains of bacteria.

To ensure a uniform understanding of what entails resistance, a bacterium is considered to be "not susceptible" when it tests as resistant, intermediate, or nonsusceptible in standardized clinical microbiological tests that have been approved by government agencies, such as the **U.S. Food and Drug Administration (FDA)**, the **Clinical & Laboratory Standards Institute (CLSI)**, or the **European Committee on Antimicrobial Susceptibility Testing (EUCAST)**. Most clinical microbiology laboratories test a broad panel of antibiotics, with representatives from several different antibiotic categories that are typically used as first- or second-line drugs for treating infections. However, there is a practical limitation on the number of antibiotics that can be routinely and inexpensively tested at any given time. More and more the results from the first-test panel are coming back with indications that the isolated bacterium might be MDR. When that happens, the laboratory must carry out additional testing using supplemental panels of drugs in order to find drugs effective against the bacterial isolate, which delays diagnosis and increases overall health care costs to treat MDR infections (see Box 16-1).

Until recently, these MDR pathogens were limited mostly to hospitals or other health care-associated settings, such as nursing homes, outpatient-care centers, and day cares. Indeed, there is a group of MDR pathogens, comprised of both Gram-negative and Gram-positive bacteria that are notorious for causing nosocomial infections. These MDR pathogens have evolved multiple mechanisms for escaping antibiotics and have been dubbed "**ESKAPE**" pathogens, with the acronym coming from the first letter of their names: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. These ESKAPE pathogens are placing an enormous financial and resource burden on global health care. Unfortunately, these ESKAPE pathogens are also being observed with community-acquired infections.

Although MDR literally means "resistant to more than one drug," in practice most scientists in the field consider bacteria to be MDR if they test as being resistant to more than two categories of antibiotics, based on *in vitro* antibiotic susceptibility tests. Thus, by the currently accepted definition, MDR bacteria are not susceptible to at least one approved drug in three or more antibiotic categories. This definition assumes that resistance of a bacterium to one drug in a particular antibiotic category is a good indicator of the susceptibility of that bacterium to other drugs within that same category. For instance, an *Escherichia coli* strain that is resistant to kanamycin (an aminoglycoside targeting protein synthesis), ampicillin (a penicillin-like β-lactam targeting cell wall biosynthesis), and tetracycline (a polyketide targeting protein synthesis) is considered MDR because it is not susceptible to three structurally diverse categories of antibiotics, even though two of them have the same bacterial site of action (protein synthesis). In another example, a MRSA (methicillin-resistant *S. aureus*) isolate that is resistant to oxacillin (a first-generation β-lactam similar to methicillin that is resistant to penicillinase), cefazolin (a first-generation cephalosporin), cefoxitin (a second-generation cephalosporin), clavulanic acid (a suicide-substrate inhibitor of β-lactamase), and imipenem (a first-generation carbapenem) predicts that this isolate is resistant to all categories of β-lactams, including all members of the categories of penicillins, cephalosporins, clavulanic acid-like inhibitors, and carbapenems, and thus is also considered MDR, even though the primary site of action (cell wall biosynthesis) is the same.

Bacterial isolates deemed MDR often have many different resistance profiles. For example, two clinical *E. coli* isolates may both be considered MDR even though one is resistant to trimethoprim-sulfamethoxazole (a drug combination targeting tetrahydrofolate biosynthesis), cefazolin (a first-generation cephalosporin targeting cell wall biosynthesis), and ciprofloxacin (a fluoroquinolone targeting DNA gyrase), while the other is resistant to imipenem (a first-generation carbapenem targeting cell wall biosynthesis), gentamicin (an aminoglycoside targeting protein synthesis) and tigecycline (a third-generation tetracycline targeting protein synthesis). This high diversity in MDR profiles places a huge financial, time, and resource burden on clinical microbiological laboratories to rapidly provide physicians with recommendations for interventions.

Although bacterial culture methods for evaluating antibiotic susceptibility profiles remain the gold standard for most clinical microbiological laboratories, the advent of multiplex PCR analysis and next-generation high-throughput metagenomic sequencing technologies (see chapter 5) are beginning to be applied to tackle the daunting task of rapidly identifying and monitoring antibiotic resistance profiles of MDR bacteria. Indeed, the rapidly expanding genome sequencing data that is accumulating from MDR bacteria has spurred the creation of several publicly available database resources, such as the **ARDB (Antibiotic Resistance Genes Database)**, the **CARD (Comprehensive Antibiotic Resistance Database)**, **ResFinder (Resistance Finder)**, and **Resfams (Resistance Families)**, which provide centralized compilations of known antibiotic-resistance genes and bioinformatic tools to facilitate gene annotation and detection of mutations associated with antibiotic resistance.

Unlike culture-based methods, metagenomic sequencing methods provide quantitative information about the complete MDR bacterial burden (the so-called **resistome**) relative to other microbiota in the microbial community and could potentially identify patients who are at increased risk for MDR infection, such as those given a particular antibiotic regimen or exposed to an invasive MDR pathogen. For example, metagenomic sequence profiling can be used to monitor the resistome of a hospitalized patient in an intensive care unit to gauge susceptibility and possible types of interventions that could be used, should they arise (Figure 16-1).

Multiple Resistance and Genetic Linkage

The first resistance mechanisms to be described conferred resistance to a single class of antibiotics. For example, β-lactamases confer resistance to members of the penicillin-cephalosporin family but not to protein synthesis inhibitors. There are two types of exceptions to this rule, which can lead to MDR. The first is the **multidrug efflux pumps**, pumps that excrete antibiotics of more than one type (more on this later). Current structural work is aimed at addressing how such pumps are nonspecific for more than one antibiotic class, whereas they do not export small molecules essential to the bacterium. The second exception is the macrolide-streptogramin-lincosamide type of erythromycin resistance, which makes a bacterium resistant to three different classes of antibiotics. This type of resistance is possible because these three types of antibiotics bind to overlapping sites near the mouth of the exit tunnel of bacterial ribosomes.

Another distinct mechanism leading to the development of MDR bacteria can occur through genetically linked resistance genes. These genetic linkages can develop when two or more resistance genes are acquired by a single plasmid. Resistance genes can move onto plasmids via transposons and integrons (described later in this chapter). In bacterial strains where genetically linked resistance gene clusters occur, selection by one class of antibiotic can also retain the resistance genes that confer resistance to unrelated antibiotics. Therefore, if a plasmid contains both a tetracycline-resistance gene and a macrolide-resistance gene, exposure of that strain to tetracycline not only selects for maintenance of the tetracyclin-resistance gene, but also for maintenance of the erythromycin-resistance gene.

Many physicians have assumed that use of a particular class of antibiotic only selects for resistance to that particular class of antibiotic. If this were true, cessation of use of that antibiotic should allow resistance to that antibiotic to decrease. The presence of MDR genes and the genetic linkages of multiple resistance genes ensure that this desirable outcome will not occur in many cases due to cross-selection. To make matters worse, there are some cases in which disinfectant-resistance genes have proved to be linked genetically to antibiotic-resistance genes. In such cases, disinfectant use could select for the maintenance of the antibiotic-resistance genes. Whereas we had all hoped that disinfectants and antiseptics would help protect us from antibiotic-resistant bacteria, they may in some cases have exactly the opposite effect. Moreover, cases of multiple resistance and genetic linkage are becoming more common. Perhaps the best example of this alarming trend is the escalating spread of MRSA strains, which can be acquired in the community as well as in hospitals. MRSA strains, which were initially identified for their resistance to the β-lactam methicillin, are now often resistant to multiple antibiotics besides β-lactams, including macrolides, tetracycline, aminoglycosides, and antiseptics.

Next-Generation MDR Pathogens: The "Superbugs"!

Acknowledging that bacteria can have different degrees of MDR, the terms **extensively drug resistant (XDR)** and **pan-drug resistant (PDR)** have now been introduced. By definition, XDR bacteria are not susceptible to at least one

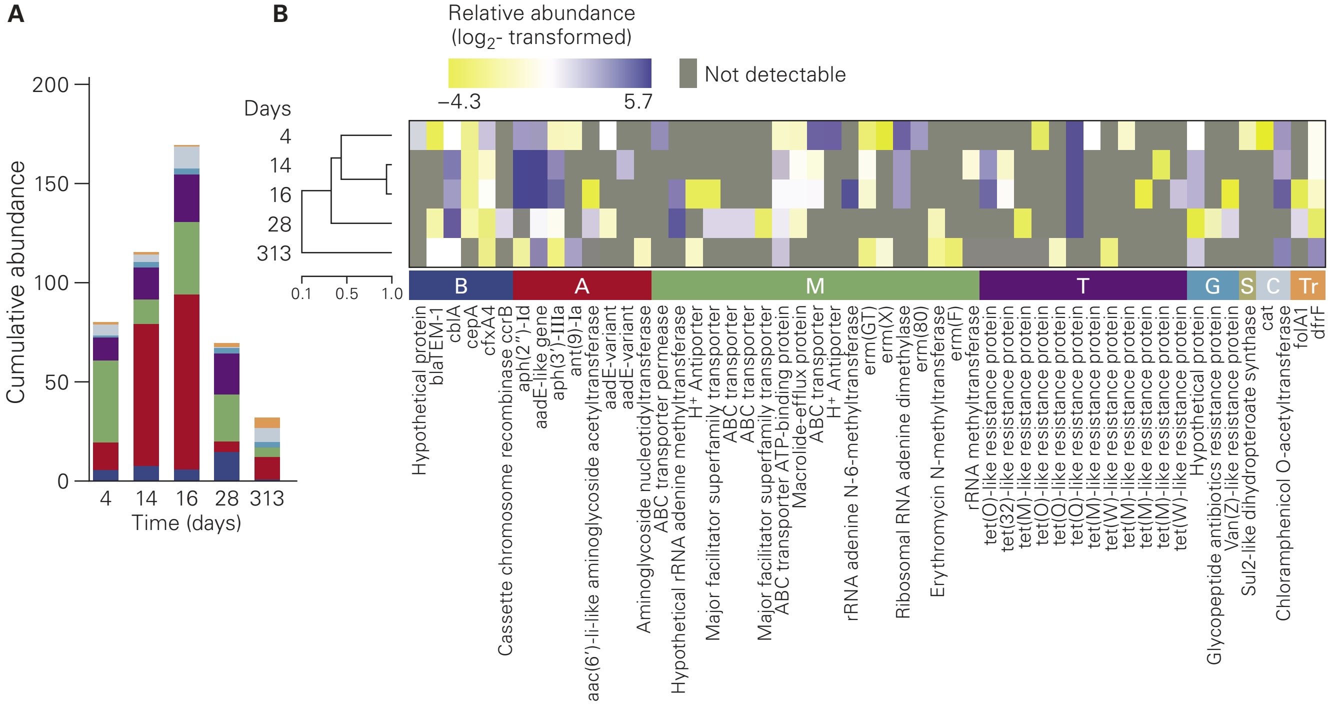

Figure 16-1. Resistome dynamics in a patient during and after hospitalization. Metagenomic sequencing analysis of the fecal resistome of a patient was performed on fecal samples during a hospital stay in the intensive care ward (days 4, 14, and 16), further hospitalization in a medium care ward (day 28), and 270 days after hospital discharge (day 313). The resistance gene families are indicated by the colored bars: A, aminoglycosides; B, β-lactams; C, chloramphenicols; G, glycopeptides; M, macrolides; S, sulfonamides; T, tetracyclines; Tr, trimethoprim. **(A)** The cumulative abundances of antibiotic-resistance genes for each family were determined from metagenomic analysis of each sample. **(B)** The heat map indicates the relative abundance of antibiotic-resistance genes that were present in the patient's gut microbiota during and after hospitalization. The dendrogram shows the relative similarity of the samples to each other. The results showed that resistance to aminoglycosides expanded during the ICU stay, most likely due to treatment with tobramycin, which was used to prophylactically treat the patient to prevent infection with opportunistic pathogens. The increased levels of macrolide-resistance genes were likely due to use of erythromycin, which was used to help with gastric emptying during the ICU stay. The high prevalence of tetracycline resistance was similar to that observed in other healthy individuals and may reflect the naturally occurring abundance of these genes in the normal population. Notably, although the prevalence of antibiotic-resistance genes in the normal microbiota of the gut after hospitalization decreased, the genes remained detectable long afterward. Adapted from Buelow E, Gonzalez TB, Versluis D, Oostdijk EA, Ogilvie LA, van Mourik MS, Oosterink E, van Passel MW, Smidt H, D'Andrea MM, de Been M, Jones BV, Willems RJ, Bonten MJ, van Schaik W. 2014. *J Antimicrob Chemother* **69:**2215–2223, with permission.

drug in all but two or fewer antibiotic categories, while PDR bacteria are not susceptible to any of the approved drugs in all antibiotic categories. Particularly problematic is the emergence of XDR ESKAPE pathogens that are resistant to fluoroquinolones, rifampin, and carbapenems, previously considered the preferred drugs of last resort. In many public health and media circles, pathogenic bacteria classified as XDR or PDR have been called **superbugs** for their incredible resistance to most or all currently available antibiotics. For an example of an ESKAPE pathogen that has recently emerged as an XDR/PDR "superbug" and has the health care community trembling in its boots, see Box 16-2. The recent discovery of the colistin-resistance gene, *mcr-1*, in *E. coli* have many in the field fearing that this may herald the inevitable emergence of additional PDR superbugs, should XDR strains of *E. coli* and other ESKAPE pathogens also acquire the *mcr-1* gene.

Since *Mycobacterium tuberculosis* (Mtb) is inherently resistant to most antibiotics, MDR-Mtb usually refers to *M. tuberculosis* strains that are resistant to the two most powerful first-line anti-Mtb drugs, isoniazid and rifampin. Treatment of infections with MDR-Mtb requires simultaneous use of up to five of the second-line anti-Mtb drugs, including pyrazinamide (a prodrug that gets converted into an inhibitor of fatty acid synthase I), ethambutol (an inhibitor of biosynthesis of arabinogalactan that forms part of the mycobacterial cell wall), fluoroquinolones (ciprofloxacin and moxifloxacin that inhibit DNA gyrase), aminoglycosides (amikacin and kanamycin that inhibit protein synthesis), rifabutin (semisynthetic rifampin that inhibits DNA-dependent RNA polymerase), streptomycin (an aminoglycoside that inhibits protein synthesis), or cycloserine (which mimics D-alanine to block cell wall synthesis). These second-line drugs are less effective, more toxic, and more expensive.

Box 16-2.

A New Biothreat Emerges from the Middle East

Shortly after Operation Iraqi Freedom launched in 2003, an alarming number of combat-injured soldiers, who were being transported to military hospitals first in Germany and then in the United States, were succumbing to a mysterious deadly pathogen, the Gram-negative bacterium *Acinetobacter baumannii*. U.S. soldiers injured in the field from gunfire or improvised explosive devices, even when those injuries were initially considered non-life-threatening, were dying (with mortality rates of 25%) from infection with new highly virulent and multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains of *A. baumannii*. Additional fatalities were also being reported among Canadian and British armed forces.

Prior to 2003, this bacterium was only encountered as a relatively uncommon opportunistic pathogen, but since then it has spread at an alarming rate, first in military and now also in nonmilitary hospital settings all around the world. Indeed, with the increasing incidence and growing prevalence, this "superbug" has emerged as a major global health threat. Many clinical isolates of *A. baumannii* show extensive or pan-resistance to all or nearly all classes of antibiotics (see table), such that only a few treatment options remain.

The nosocomial spread of these MDR or XDR strains in patients with other confounding illnesses increases the mortality rates up to 50%. To make matters worse, *A. baumannii* can survive on environmental surfaces for months, readily adhering to both biological and abiotic surfaces, on which it forms biofilms, thereby making decontamination of medical equipment and facilities challenging and extremely expensive.

Sources:

Jones A, Morgan D, Walsh A, Turton J, Livermore D, Pitt T, Green A, Gill M, Mortiboy D. 2006. Importation of multidrug-resistant *Acinetobacter* spp infections with casualties from Iraq. *Lancet Infect Dis* **6:**317–318.

McConnell MJ, Actis L, Pachón J. 2013. *Acinetobacter baumannii*: human infections, factors contributing to pathogenesis and animal models. *FEMS Microbiol Rev* **37:**130–155.

Major resistance mechanisms found in *Acinetobacter baumannii*[a]

DRUG CLASS	RESISTANCE MECHANISM	EXAMPLES
β-lactams	Inactivating enzymes	β-lactamases (AmpC, TEM, VEB, PER, CTX-M, SHV)
		Carbapenemases (OXA-23, -40, -51, -58, -143-like; VIM; IMP; NDM-1, -2)
	Decreased outer membrane protein expression	CarO, 33–36 kDa protein, OprD-like protein
	Altered penicillin-binding protein expression	PBP2
	Efflux pumps	AdeABC
Fluoroquinolones	Target modification	Mutations in *gyrA* and *parC*
	Efflux pumps	AdeABC, AdeM
Amino glycosides	Aminoglycoside modifying enzymes	AAC, ANT, APH
	Efflux pumps	AdeABC, AdeM
	Ribosomal methylation	ArmA
Tetracyclines	Efflux pumps	AdeABC, TetA, TetB
	Ribosomal protection	TetM
Glycylcyclines	Efflux pumps	AdeABC
Polymyxins (colistin)	Target modification	Mutations in the PmrA/B two-component system (LPS modification), mutations in LPS biosynthesis genes

[a] Reproduced from **McConnell MJ, Actis L, Pachón J.** 2013. *FEMS Microbiol Rev* **37:**130–155, with permission.

They also require extended treatment times with a minimum of six months but more typically nine months, and sometimes up to two years. Even with a nine-month treatment regimen, the cure rates for MDR-Mtb infections are only 70% to 90%, depending on the particular combination of second-line antibiotics used.

Antibiotics must be used in combination to be effective against Mtb. To appreciate why this is the case, one can consider that a well-contained Mtb granuloma in an immunocompetent individual usually has about 10^2 to 10^4 bacteria, while an active lesion with growing bacteria contains 10^7 to 10^9 bacteria. Given that all known drug resistances in Mtb occur through chromosomal mutation, an important factor that contributes to gaining multiple resistances is the rate at which Mtb mutates. Even though Mtb replicates very slowly, it is able to accumulate mutations within the lesions such that resistance against any one of the antibiotics can occur within an active lesion. Typical frequencies (mutation rates) at which Mtb gains resistance for the usual line of antibiotics are: isoniazid at 10^{-8}, rifampin at 10^{-9}, ethambutol at 10^{-7}, pyrazinamide at 10^{-5}, and streptomycin at 10^{-8}. At these mutation rates, the likelihood that a mutation resulting in resistance against one antibiotic will occur within a lesion is fairly high. However, the likelihood that two mutations against two different antibiotics administered concurrently will occur within the same lesion is far less. For instance, the likelihood that resistance against both isoniazid and rifampin will occur within the same bacterium in a lesion is $10^{-8} \times 10^{-9} = 10^{-17}$. Adding an additional drug to the regimen should reduce the likelihood of bacteria surviving treatment with all three drugs even more.

Given that most Mtb treatment regimens involve concurrent administration of multiple drugs, how is it that the prevalence of MDR-Mtb strains is increasing at such a troubling rate? The most common factors cited for treatment failure due to MDR are: (i) patient noncompliance due to the long treatment regimen, adverse drug side effects, and/or the expense of monitoring to ensure compliance; (ii) physician prescription of inappropriate drug regimens and/or dosages; (iii) use of poor quality or expired drugs, particularly in developing countries; and (iv) primary infection with Mtb strains that are already MDR.

XDR-Mtb occurs when the MDR-Mtb strain is also resistant to fluoroquinolone and at least one of the other second-line drugs. Ominously, the first cases of PDR-Mtb, also referred to as **totally drug-resistant Mtb (TDR-Mtb)**, have already been reported in patients from several parts of the world, including Iran, South Africa, Italy, and India. These TDR/PDR-Mtb strains are resistant to all first- and second-line anti-Mtb drugs, such that there are no viable treatment options left for these patients, resulting in very high fatality rates.

Mechanisms of Antibiotic Resistance

Overview of Resistance Mechanisms

Mechanisms of antibiotic resistance can be grouped into four main categories. One is restricted access of the antibiotic to its target. For example, the outer membrane of Gram-negative bacteria can serve as an effective barrier against certain antibiotics, and this resistance can be enhanced by changes in the outer membrane properties that allow the bacteria to avoid taking up antibiotics. Another way to restrict access is to prevent the antibiotic from accumulating to high-inhibitory concentrations in the cell by increasing active efflux (pumping out) of the drug from the bacteria. Efflux pumps are ubiquitous in Gram-negative and Gram-positive bacteria. A second category of resistance mechanisms is enzymes that inactivate or chemically modify the antibiotic, either by hydrolyzing it or by adding chemical groups to some important part of the antibiotic that interferes with binding of the drug to its target. A third category is modification of the antibiotic target. In this type of resistance, the bacteria accumulate mutations in a gene encoding a target protein or rRNA or acquire proteins that modify the target, such that the target protein still works but no longer binds the antibiotic strongly enough to cause inhibition. In the fourth category, failure to activate the antibiotic, mutations can occur that decrease the expression of an enzyme that activates the antibiotic. If the bacteria do not activate the antibiotic, it is harmless.

An interesting feature of many resistance mechanisms is that the proteins that mediate them are often related to bacterial housekeeping proteins. For example, some enzymes that inactivate penicillin are related to and may have evolved from the transpeptidase enzymes that carry out the cross-linking of peptidoglycan (see chapter 15), the enzymes that are inactivated by penicillin. Apparently, bacteria sometimes adapt the target of an antibiotic to become an offensive weapon against antibiotics. Although the different types of resistance mechanisms will be considered individually here, it is important to realize that bacteria can combine more than one mechanism of resistance to increase their defensive shield against an antibiotic. As a result, MDR/XDR bacteria contain multiple, and often separate, mechanisms that impart resistance to several different classes of antibiotics, and in some cases the resistances act not just additively, but also synergistically.

Resistance to Antiseptics and Disinfectants

Resistance to antiseptics and disinfectants is poorly understood in general, but some resistance mechanisms are known. Many antiseptics and disinfectants, especially those such as quaternary ammonium compounds (QACs, see Table 15-2) that attack membranes, are less effective against Gram-negative bacteria than Gram-positive bacteria. The reason seems to be that lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria prevents hydrophobic molecules from intercalating into the outer membrane, while outer membrane porins restrict access to the cytoplasmic membrane by limiting diffusion into the periplasm. Some membrane-active antiseptics, however, can breach the barrier posed by the outer membrane.

An interesting type of resistance to QACs has been found in staphylococci and *Listeria*: a cytoplasmic membrane efflux pump that pumps the QAC out of the cell cytoplasm. Why this would make the bacteria resistant to QACs, which are thought to act mainly by dissolving membranes, is still unclear, but these pumps are fairly effective in protecting the bacteria from QACs. Genes encoding these QAC efflux pumps have been found on plasmids as well as on transposons and putative mobile elements in the chromosome. The fact that resistance to antiseptics and disinfectants can develop is a disturbing discovery, because these compounds have been considered an important first line of defense in preventing bacterial infections.

Limiting Access of the Antibiotic

Antibiotics must first reach their target in order to have an effect. An effective way to block the action of an antibiotic is to restrict the access of the antibiotic to its target by not letting it get into the cell. Some bacteria are inherently resistant to certain chemicals because the compounds cannot penetrate through their outer surfaces. Other bacteria can alter their cell surfaces through the mutation of genes that results in changes in surface charges or compositions of their membrane phospholipids or cell walls. For instance, mycobacteria have an unusual mycolic acid cell wall that is difficult for many antibiotics to penetrate.

The outer membrane of Gram-negative bacteria also serves as such a barrier for many drugs, and these bacteria can circumvent antibiotic penetration by changing their membrane properties or by changing the pore size of porins in the outer membrane. Another way to limit access is to actively remove the antibiotic once it has entered the cell, so that the antibiotic is not allowed to accumulate to high enough concentrations that could inhibit bacterial function.

Outer Membrane Alterations. Some bacteria can alter their outer surfaces to exclude antibiotics that have certain charges. For instance, some strains of the Gram-positive *Enterococcus faecium* have acquired positive charges on their surfaces that electrostatically repulse positively charged antibiotics, such as the active daptomycin-Ca^{2+} complex that normally forms on the cell surface of susceptible bacteria. Since resistance against vancomycin (the previous drug of last resort) in clinical isolates of *E. faecium* has now surpassed 80%, conferring resistance against daptomycin, the current antibiotic of last resort, makes treatment of vancomycin-resistant MDR strains of *E. faecium* a daunting clinical challenge.

Outer Membrane Porins. β-Lactam antibiotics must transit the Gram-negative outer membrane to reach the periplasm where the penicillin-binding proteins are located. Other types of antibiotics that have targets in the bacterial cytoplasm must be transported across the cytoplasmic membrane. In Gram-negative bacteria, the outer membrane can function as a barrier to antibiotic entry. The reason vancomycin, which is very effective against Gram-positive bacteria, is not as effective against many Gram-negative bacteria is that it is too bulky to diffuse through the outer membrane porin proteins of those bacteria.

Porin proteins form β-barrel structures in the outer membrane that allow the selective diffusion of small molecules into the periplasm. The genomes of Gram-negative bacteria encode many different porins with a variety of permeability limits, and changing stress conditions regulates the expression of porin genes. For example, *P. aeruginosa,* an organism that causes eye infections associated with improper contact lens use, bacteremia in burn patients, and lung infections in cystic fibrosis patients, has a large genome that encodes roughly 5,500 genes, of which over 70 encode porin proteins from three different structural families. Clearly, *P. aeruginosa* has considerable capacity to modulate the uptake of different-sized molecules by its outer membrane.

Mutations in genes encoding porins can increase the permeability of the outer membrane to bulky compounds and thereby confer sensitivity of Gram-negative bacteria to vancomycin and other bulky antibiotics. Conversely, bacteria can accumulate mutations that further restrict the diffusion of large antibiotics through the outer membrane and thereby increase resistance. Since some types of porins are relatively nonselective, a single porin mutation can confer resistance to more than one type of antibiotic. For a long time, the importance of mutations in porins as a mechanism of resistance was underappreciated because this type of resistance usually confers increases in resistance, as measured in terms of minimal inhibitory concentrations (MIC), of only five- to tenfold. In contrast, other resistance mechanisms can confer greater than fifty- to a hundredfold increases in MIC. However, in clinical settings where the highest concentration of antibiotic achievable at the site of infection is sometimes less than five times higher than the level required to kill or inhibit growth of the bacteria, a tenfold increase in MIC can be as disastrous as a hundredfold increase.

Reduced Uptake Across the Cytoplasmic Membrane. Penicillin and other β-lactam antibiotics do not need to reach the cytoplasm because they act on targets at bacterial extracellular surfaces or in the periplasm. However, other antibiotics must be taken up into the bacterial cell to exert their action. These antibiotics, such as the aminoglycosides, use specific transporters to enter bacterial cells. An obvious way for bacteria to resist the action of such an antibiotic that has a target in the bacterial cytoplasm (e.g., the ribosome or DNA gyrase) is to fail to transport the antibiotic across the cytoplasmic membrane, yet this does not seem to be a common mechanism of resistance. In some cases, the lack of such a mechanism of resistance is understandable. Tetracycline, for example, has a transporter (the cation/proton antiporter TetA in *E. coli*) that actively transports the antibiotic into the bacterial cell using proton motive force, but tetracycline also can diffuse readily through membranes because it is a hydrophobic compound. Thus, mutations in TetA will not necessarily lead to resistance.

However, resistance to more polar antibiotics like aminoglycosides still does not readily appear by accumulation of mutations in their transporter genes. There are several possible reasons for this observation: one possibility is that this transporter might be essential for bacterial survival; another is that there may be multiple redundant transporters that can take up this class of antibiotic. An interesting observation is that some bacteria become much more resistant to aminoglycosides when they are growing under anaerobic conditions. Indeed, *E. coli* becomes almost 10 times more resistant to aminoglycosides when growing anaerobically, and many anaerobes are totally resistant to aminoglycosides. The reason appears to be that the expression and activity of the transporter is oxygen-dependent.

Active Efflux of Antibiotics. Efflux pumps are membrane proteins that use energy to pump small molecules out of the bacterial cytoplasm. If this small molecule is an antibiotic, resistance results because the antibiotic is prevented from reaching a high enough concentration within the cytoplasm to be effective at inhibiting its target. Bacteria contain multiple efflux pumps, many of which can contribute to reducing cytoplasmic concentrations of antibiotics. For example, the genomes of *E. coli* and *P. aeruginosa* each encode over 30 different efflux pumps. These efflux pumps normally play roles in maintaining homeostasis through the uptake of nutrient metabolites or metal ions while pumping toxic substances and metals out of the bacterial cell. Many efflux pumps move horizontally between different bacterial pathogens on mobile genetic elements, such as plasmids or transposons, and are frequently part of pathogenicity islands.

Efflux pumps fall into two general categories that are made up of five protein structural families (Figure 16-2). The majority of the efflux pumps are **antiporters** that use the uptake of protons (H^+) or sodium ions (Na^+) as the source of energy to pump the antibiotics and other small molecules from the inside of the cell to the outside. Members of the resistance-nodulation-division (RND), the major facilitator superfamily (MFS), and the small multidrug resistance (SMR) protein families are H^+ antiporters, while members of the multidrug and toxic compound extrusion (MATE) protein family are often Na^+ antiporters. **ABC transporters** belong to the ATP-binding cassette (ABC) structural superfamily of membrane transport proteins and use ATP hydrolysis to pump the drug out. Recall, we encountered members of this superfamily in chapter 13 as part of the Sec-independent type 1 secretion system (T1SS, see Figure 13-5).

Efflux pumps of the MFS, SMR, MATE, and ABC families are found widely in both Gram-positive and Gram-negative bacteria. In contrast, the major clinically relevant efflux pumps of Gram-negative bacteria belong to the RND structural family, consisting of multisubunit proteins that span the inner membrane, periplasm, and outer membrane to channel the antibiotic from inside the cell or from the periplasm to outside the cell. In clinical isolates, mutations conferring increased antibiotic resistance are often found in the promoter regions of the operons encoding RND antiporter proteins, resulting in overexpression of the efflux pumps and enhanced antibiotic resistance.

The first efflux pump mechanism to be characterized extensively mediates resistance to tetracyclines. The resistance protein is an antiporter pump (called TetA, TetB, TetC, etc., in different bacteria) located in the cytoplasmic membrane. These efflux pumps, encoded by genes on the chromosome, plasmids, or other transmissible elements, are members of the MFS structural family that catalyze H^+-dependent transport of tetracycline out of the bacterium. Since tetracycline is removed as rapidly as it is taken up, the intracellular concentration of tetracycline remains too low to effectively inhibit protein synthesis.

Efflux pumps have now been discovered for nearly every class of antibiotic, and they cause serious clinical problems by imparting resistance directly or indirectly to a wide range of antibiotic categories, including β-lactams, macrolides, fluoroquinolones, streptogramins, and tetracyclines. Some efflux pumps are highly specific for a particular compound (e.g., the TetA efflux pump only transports tetracycline), whereas others pump out many different types of compounds (e.g., tetracyclines and macrolides), resulting in resistance to multiple antibiotics. Efflux pumps consequently represent an extremely serious form of MDR mechanism that is challenging to overcome. For instance, *P. aeruginosa* and *A. baumannii* possess a large number of efflux pumps with broad specificity that facilitate removal of all antibiotics from certain classes, such as tetracyclines and macrolides. Indeed, most XDR strains have many of these efflux pumps as part of their arsenal.

Enzymatic Inactivation of the Antibiotic

Bacteria can gain antibiotic resistance by producing enzymes that inactivate antibiotics via hydrolytic cleavage or by modification of functional groups that reduce binding interactions of the antibiotic with the bacterial target. Examples of this type of resistance are the enzymatic hydrolysis of the β-lactam ring in penicillin-related antibiotics through the action of β-lactamases and the covalent modification of chloramphenicol through acetylation of one or two of its hydroxyl (–OH) groups, which sterically prevent them from binding to their targets. Because these resistances are mediated through the action of a single enzyme, the genes encoding these proteins are readily transmitted through HGT, leading to rapid spread among different bacteria.

β-Lactamases. A major mechanism of resistance to β-lactam antibiotics is the production of **β-lactamases**, enzymes that cleave the β-lactam ring and render the antibiotic inactive (recall Figures 15-8 and 15-27). In the serine class of β-lactamases, the –OH group of an active-site serine residue attacks the β-lactam ring, opening the ring and forming a covalent bond analogous to that formed during the catalytic reaction of transpeptidases (penicillin-binding proteins). However, unlike the transpeptidases, which preserve this covalent bond, β-lactamases allow water molecules to attack it, thereby converting the antibiotic into an inactivated form with an opened β-lactam ring and freeing the β-lactamase for another round of catalysis (see Figure 15-27).

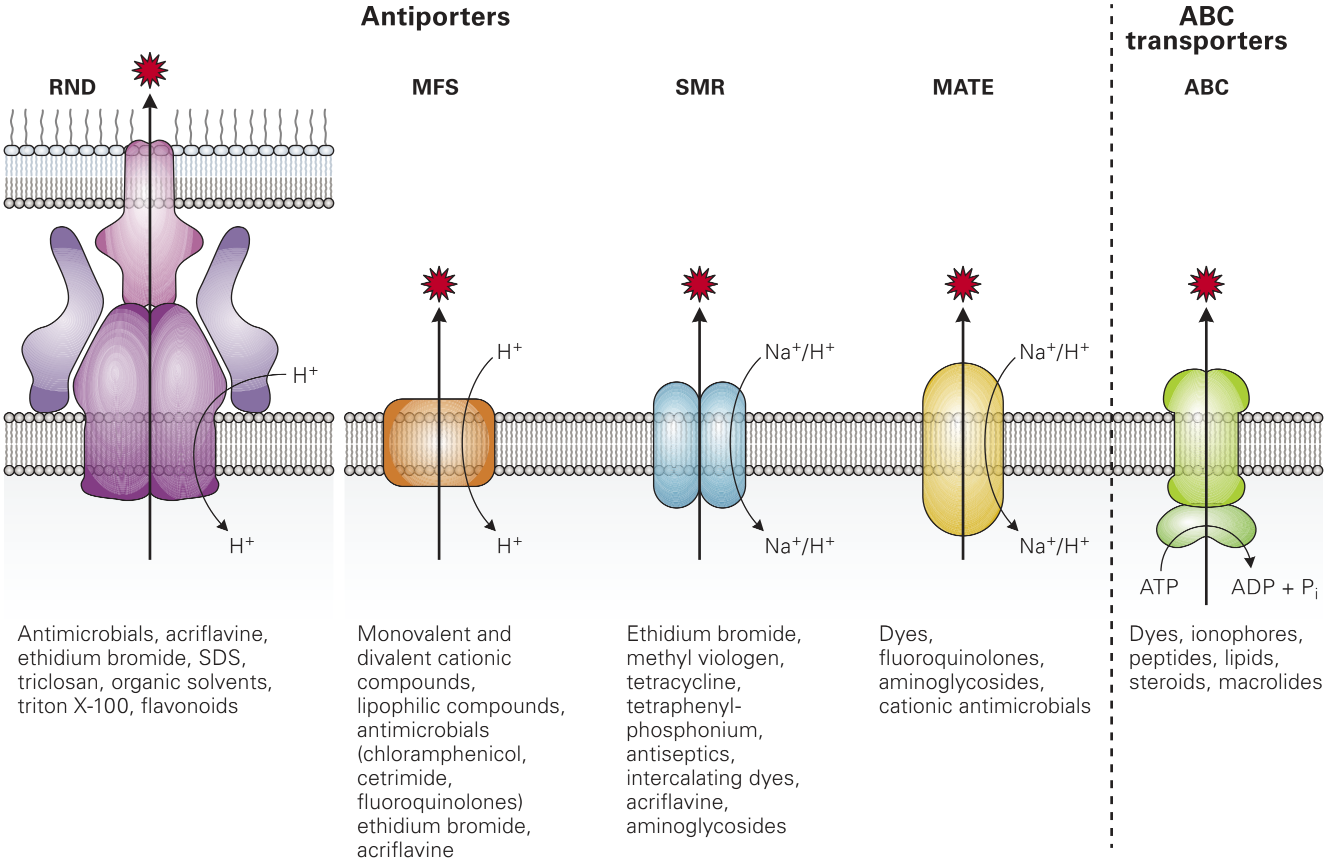
Antiporters
ABC transporters
RND
MFS
SMR
MATE
ABC
H+
H+
H+
H+
Na+/H+
Na+/H+
Na+/H+
Na+/H+
ATP
ADP + Pi
Antimicrobials, acriflavine, ethidium bromide, SDS, triclosan, organic solvents, triton X-100, flavonoids
Monovalent and divalent cationic compounds, lipophilic compounds, antimicrobials (chloramphenicol, cetrimide, fluoroquinolones) ethidium bromide, acriflavine
Ethidium bromide, methyl viologen, tetracycline, tetraphenyl-phosphonium, antiseptics, intercalating dyes, acriflavine, aminoglycosides
Dyes, fluoroquinolones, aminoglycosides, cationic antimicrobials
Dyes, ionophores, peptides, lipids, steroids, macrolides

Figure 16-2. Structural families of antibiotic efflux pumps. Shown is a comparison of the five families of drug efflux pumps that contribute to antibiotic resistance. Antiporters of the RND (resistance-nodulation-cell division), MFS (major facilitator superfamily), SMR (small multidrug resistance), and MATE (multidrug and toxic compound extrusion) structural families use the uptake of protons (H^+) or sodium ions (Na^+) as the source of energy to pump the antibiotics and other small molecules out of the cell. ABC transporters use ATP hydrolysis to power the efflux pump. Data from Li XZ, Plésiat P, Nikaido H. 2015. *Clin Microbiol Rev* **28:**337–418.

Bacteria are most susceptible to the antibiotic during cell growth, but cell lysis can release more β-lactamase into the medium, which in turn reduces the amount of active antibiotic overall. Once there is sufficient inactivation of the antibiotic in the medium by the β-lactamase, the bacteria are able to recover (Figure 16-3). To overcome this problem in practice, the antibiotic is usually given in high dosages over a prolonged period of time.

β-Lactamases are secreted into the periplasmic space by Gram-negative bacteria and into the extracellular fluid by Gram-positive bacteria. Because the Gram-negative bacteria confine their β-lactamases to the periplasm and have porins that restrict the entry of β-lactams into this region, they can achieve the same level of resistance with a lower level of enzyme than Gram-positive bacteria. Unlike porin mutations, which confer resistance to many different antibiotics, β-lactamases are much more specific and are usually active against only a subset of β-lactam antibiotics. In fact, the main reason for the large number of β-lactam antibiotics

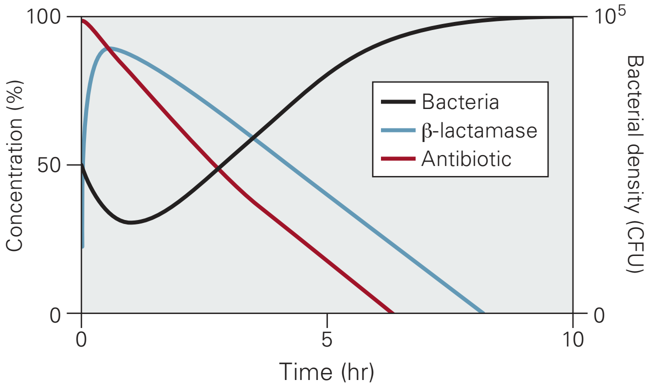

Figure 16-3. Dynamics of antibiotic-mediated bacterial cell death and the effect of β-lactamase action. Shown is a typical time course of the action of a single dose of antibiotic on bacterial growth (density) and the effect of β-lactamase that is released due to cell lysis. Bacterial recovery can only occur if there are enough bacteria present to produce and release sufficient β-lactamase that will destroy the remaining antibiotic.

now on the market is the continuing need for new β-lactam antibiotics that are not cleaved by existing β-lactamases. So far, the appearance of each new β-lactam on the market has been followed not long after by the first report of a new β-lactamase that inactivates it (hence the designation of each new class as the "next-generation" β-lactam).

Besides changing the β-lactam antibiotic, another strategy for countering β-lactamases is to mix the β-lactam antibiotic with a mechanism-based β-lactamase inhibitor, such as **clavulanic acid** (see Figure 15-27) or **sulbactam**. Clavulanic acid does not kill bacteria; instead, it prevents the β-lactamase from inactivating the antibiotic, which can then proceed to kill the bacteria. These β-lactamase inhibitors have expanded the spectrum of antibiotic utility and have enabled once again the use of some older generation β-lactams, such as ampicillin, which were in danger of becoming obsolete. However, predictably, β-lactamases have appeared that are resistant to both clavulanic acid and sulbactam inhibition. In one mechanism, a chromosomal gene encoding a β-lactamase was duplicated many times to give a much higher level of β-lactamase production, but more commonly the β-lactamase genes were carried on high-copy-number plasmids. Apparently, the excess β-lactamase produced was able to bind enough clavulanic acid to allow the remaining β-lactamase to inactivate any antibiotic present.

Since the 1980s, a new type of β-lactamase began to appear that was resistant to first- and second-generation, narrow-spectrum β-lactams and β-lactamase inhibitors, such as clavulanic acid and sulbactam, and to later-generation, extended-spectrum β-lactams, such as cephalosporins. These **extended-spectrum β-lactamases (ESBLs)** are frequently plasmid-encoded and confer resistance to a much broader set of β-lactams, severely limiting treatment options. Intravenous infusion of carbapenems, such as imipenem or ertapenem, is often the last treatment choice for MDR isolates of ESKAPE pathogens, which often carry multiple ESBLs.

A far more serious challenge to the β-lactam class of antibiotics is the appearance of **zinc-β-lactamases**. These metalloenzymes use a catalytic mechanism that does not involve active-site serine residues. Many classes of β-lactam antibiotics that are not cleaved by serine β-lactamases are avidly cleaved by zinc-β-lactamases. To make matters worse, the current β-lactamase inhibitors only inhibit the serine-β-lactamases and are completely ineffective against the zinc-β-lactamases. Importantly, many of these zinc-β-lactamases are active against the β-lactams of last resort: the carbapenems. Luckily, another inhibitor has come on the scene, called cilastatin, which can overcome carbapenemase action.

Aminoglycoside-Modifying Enzymes. The main mechanism of aminoglycoside resistance is inactivation of the antibiotic via enzymatic modification. In contrast to β-lactamases, which cleave the β-lactam ring in the antibiotic and destroy the antibiotic, aminoglycoside-modifying enzymes inactivate the antibiotic by adding functional groups (phosphoryl, adenyl, or acetyl groups) to the hydroxyl or amino groups of these antibiotics (Figure 16-4). These modifications interfere with the hydrogen-bonding network that the antibiotics use to bind tightly to the ribosome and to inhibit translation. These modifications lead to high-level resistance. In some Gram-negative species, resistance also results from inhibition of aminoglycoside uptake (see previous section).

Chloramphenicol and Streptogramin Acetyltransferases. A common mechanism of resistance to chloramphenicol is acquisition of an enzyme that adds an acetyl group to chloramphenicol (Figure 16-5A). The enzyme is called **chloramphenicol acetyltransferase (CAT)** because it transfers an acetyl group from S-adenosyl-L-methionine, a compound used in many housekeeping methyl transfer reactions, to one of the –OH groups of chloramphenicol. This acetylation prevents tight binding of chloramphenicol to the 23S rRNA peptidyltransferase site of the ribosome. Although this form of resistance is common in many bacteria, its clinical impact is somewhat limited because chloramphenicol use is restricted to an antibiotic of last resort due to serious potential side effects associated with the antibiotic, including aplastic anemia and other blood disorders.

Likewise, acetyltransferases have appeared that modify and inactivate streptogramins (Figure 16-5B). Again, the acetylation weakens the binding of the streptogramins for their targets in 23S rRNA within the ribosome. Synercid, which is a mixture of two streptogramins, had only been in use to treat human infections for a relatively short time when resistance began to be seen. Acetyltransferases that modify streptogramins are encoded by *vat* and *sat* genes of Gram-positive bacteria, such as enterococci and staphylococci. In addition, efflux by an ABC transporter pump has been found in some clinical isolates of staphylococci.

Tetracycline-Inactivating Enzymes. Several novel enzymes have been discovered that use chemical modification to inactivate tetracycline (Figure 16-6), although this form of resistance is not nearly as prevalent as that caused by efflux pumps. The ***tetX*** gene encodes an NADPH-dependent oxidoreductase that requires oxygen and NADPH to inactivate tetracycline and thus works only in aerobically growing bacteria. In this regard, it is also important to note that except for the nasopharynx and lungs, most other body sites are relatively low in free oxygen due to the fact that oxygen is tightly bound to hemoglobin. An odd feature of this resistance is that the gene was found originally in an obligate anaerobe (*Bacteroides fragilis*), despite the fact that the resistance mechanism does not work in this bacterium.

Another group of tetracycline-inactivating enzymes, dubbed **tetracycline destructases**, belongs to a superfamily of flavoenzymes that previously were not known to confer tetracycline resistance. Genes encoding these

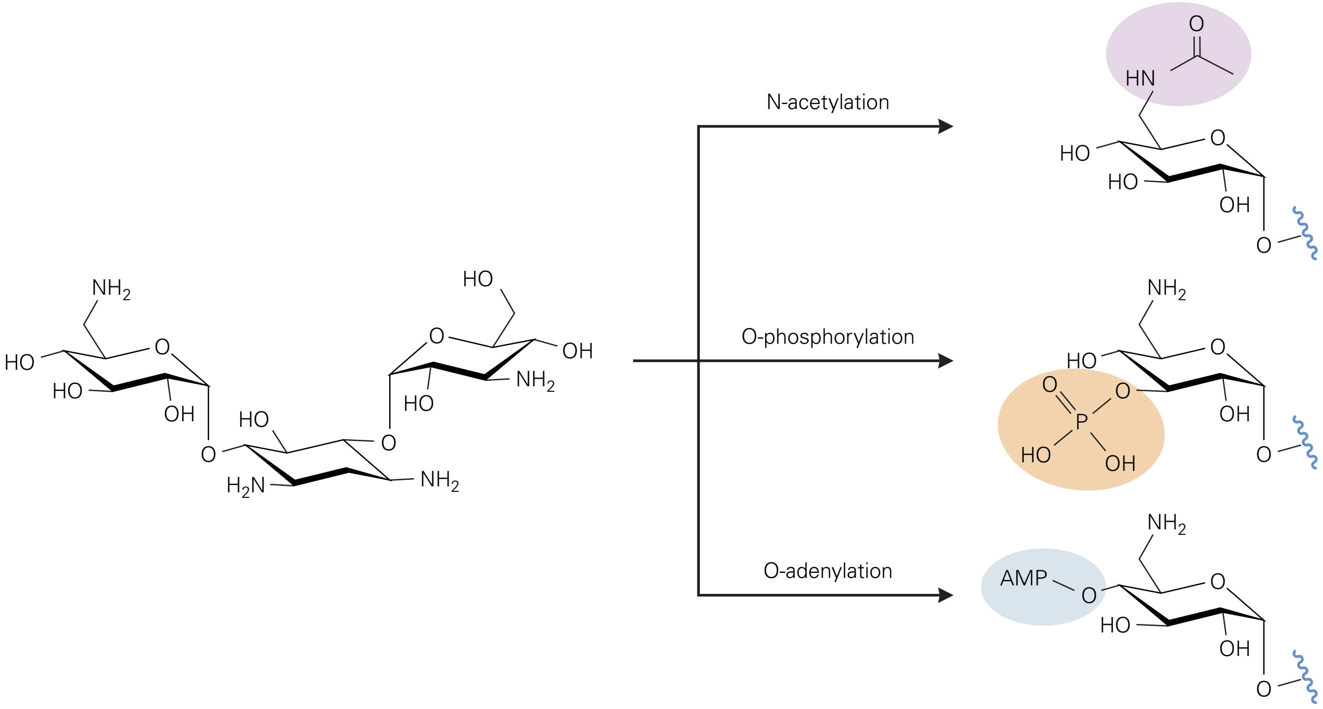

Figure 16-4. Action of aminoglycoside-inactivating enzymes. Examples of three enzymatic routes to aminoglycoside modification and deactivation: acetyl-CoA-dependent aminoglycoside acetyltransferase (AAC)-catalyzed N-acetylation of amino groups; ATP-dependent aminoglycoside phosphoryltransferase (APH)-catalyzed phosphorylation of hydroxyl groups; ATP-dependent aminoglycoside nucleotidyltransferase (ANT)-catalyzed adenylation of hydroxyl groups.

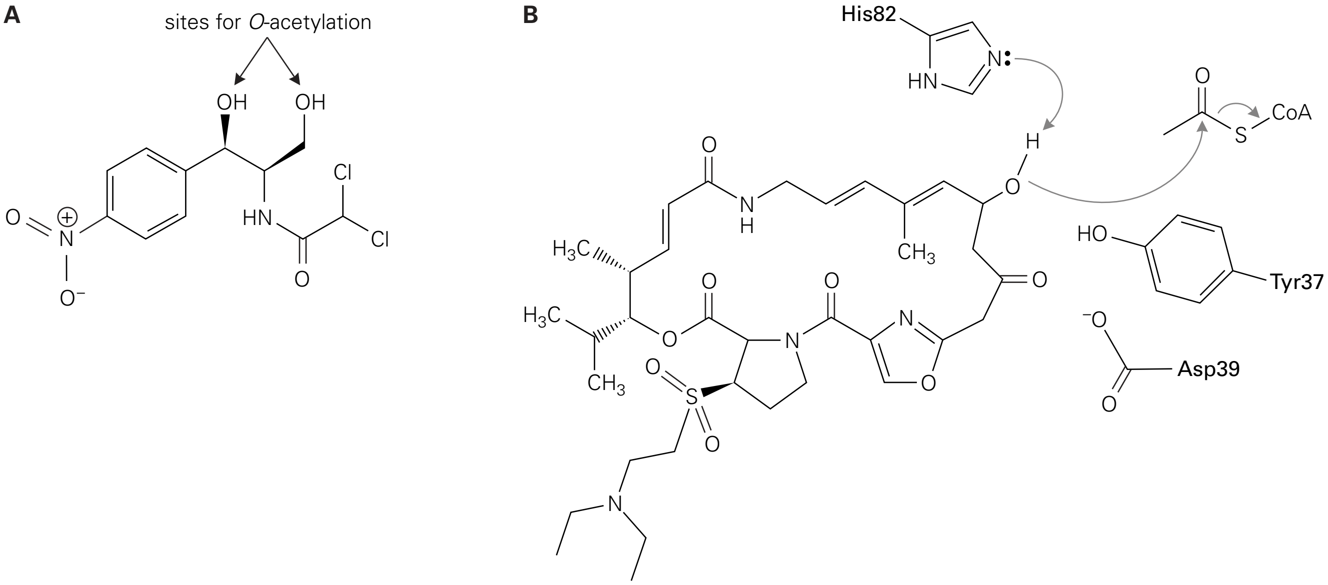

Figure 16-5. Antibiotic inactivation by acetyltransferases. (A) Chloramphenicol acetyltransferase (CAT) enzyme catalyzes the addition of acetyl groups to chloramphenicol and prevents it from binding to the 23S rRNA in the 50S subunit of bacterial ribosomes. **(B)** Reaction mechanism of acetyltransfer to streptogramin catalyzed by VatD, an acetyltransferase from *Enterococcus faecium*. The three active site residues that position the antibiotic and acetyl-CoA substrates and catalyze the transfer reaction are indicated.

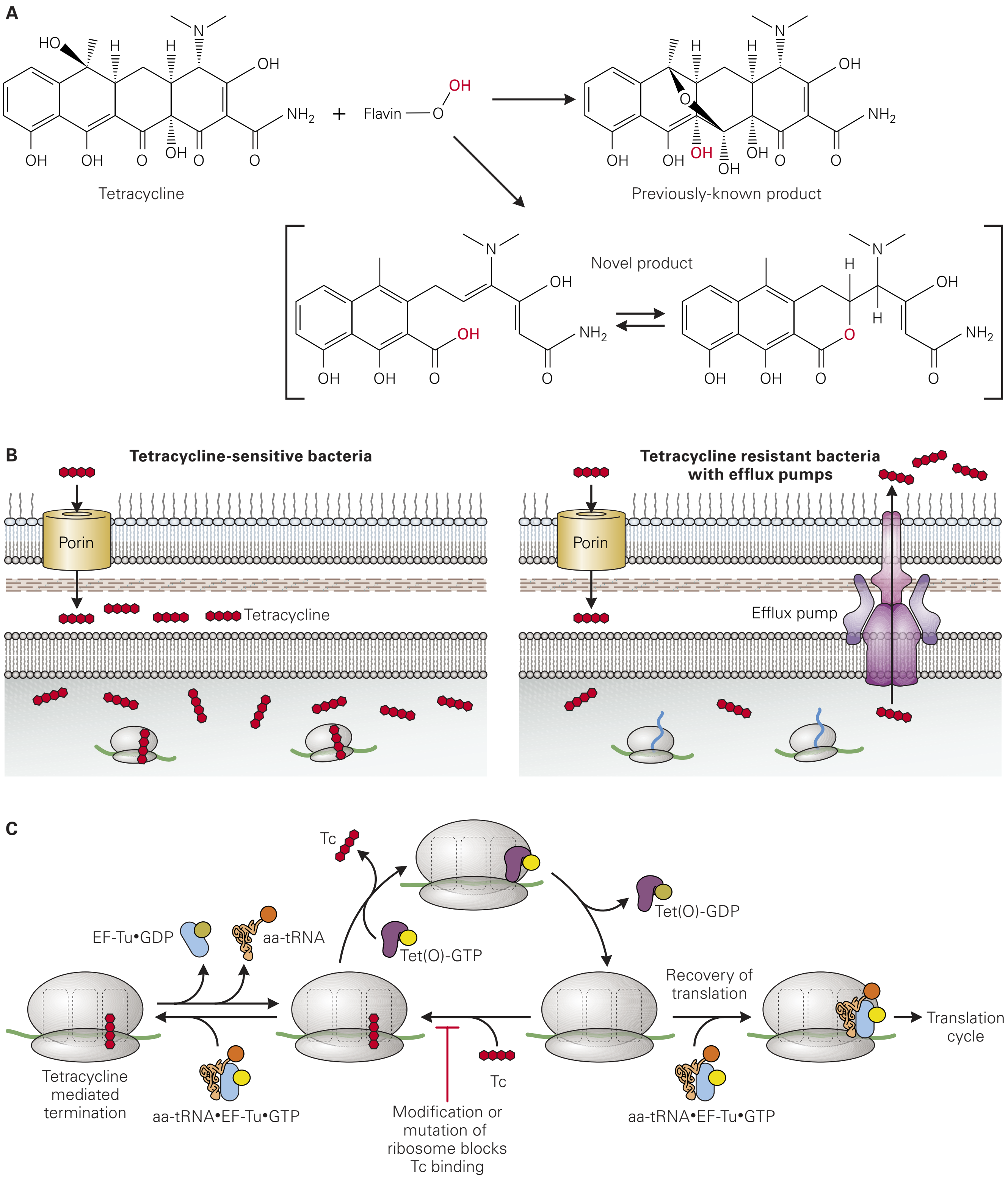
A
HO
H
H
N
OH
NH2
OH
OH
O
OH
O
O
Tetracycline
+
Flavin
O
OH
Previously-known product
Novel product
B
Tetracycline-sensitive bacteria
Porin
Tetracycline
Tetracycline resistant bacteria with efflux pumps
Porin
Efflux pump
C
Tc
Tet(O)-GDP
Tet(O)-GTP
EF-Tu•GDP
aa-tRNA
Recovery of translation
Translation cycle
Tetracycline mediated termination
aa-tRNA•EF-Tu•GTP
Tc
Modification or mutation of ribosome blocks Tc binding
aa-tRNA•EF-Tu•GTP

Figure 16-6. Mechanisms of tetracycline resistance. (A) Antibiotic modification. Tetracycline destructases are flavoenzymes that catalyze the oxidation and inactivation of tetracycline through known and novel mechanisms. Data from Forsberg KJ, Patel S, Wencewicz TA, Dantas G. 2015. *Chem Biol* **22:**888–897. **(B)** Tetracycline efflux pump. The cytoplasmic membrane protein pump (open triangle) pumps tetracycline out of the cell as fast as the transporter takes it up. Intracellular concentration of tetracycline remains too low for effective binding to ribosomes. (**C**) Ribosome modification and protection. Tetracycline (Tc) accumulation within the cell occurs and is similar to that in a sensitive cell, but the ribosome is protected through modification or mutation of the ribosome subunits, such that tetracycline no longer binds to it. Normally, tetracycline binds the 16S rRNA in the 30S ribosomal subunit and occupies the A-site where an aminoacyl-tRNA (bound to EF-Tu-GTP as a complex) would enter the A-site, thereby preventing the incoming aminoacyl-tRNA from binding productively to the codon on the mRNA resulting in Tc-mediated peptide chain termination. One type of Tc-resistance gene encodes a ribosome protection protein, such as TetO, that can rescue the ribosome from this nonproductive cycle by displacing the Tc from the ribosome in a GTP-dependent manner. TetO has structural homology to the bacterial GTPase, elongation factor EF-G. TetO binds to both the 30S and 50S ribosomal subunits near the Tc-binding site and competitively occludes Tc from binding to the ribosome. TetO then hydrolyzes the bound GTP to GDP and dissociates from the ribosome, allowing the ribosome to return to the protein translation elongation cycle.

newly discovered enzymes were isolated from various soil bacteria, and the enzymes were shown to catalyze the oxidation of tetracycline by known and novel mechanisms (Figure 16-6A). Although these genes have not yet been found in any clinical setting, a homolog was also identified in a clinical isolate of *Legionella longbeachae*. Whether these resistance mechanisms hold significant clinical relevance remains to be seen, but these findings highlight the potential for further surprises from nature and warrant further monitoring.

Modification or Protection of the Antibiotic Target

Besides modifying or destroying the antibiotic, bacteria can become resistant to several different classes of antibiotics by modifying the bacterial cell target. Target modifications can be divided into two general classes. The first class of target modifications is the accumulation of spontaneous mutations in the target that interfere with antibiotic binding. Antibiotic stress is a powerful selection condition, especially for bacteriostatic antibiotics that do not kill bacteria outright. The second class of target modifications is chemical additions or changes to the targets, such as addition of methyl groups, which impede antibiotic binding but still allow target function. Several examples of these two mechanisms are discussed in the subsequent sections.

Resistance to Macrolides. A classic example of target modification as a mechanism of antibiotic resistance is the effect that mutational changes in the nucleic acid base A at position 2058 in bacterial 23S rRNA has on sensitivity to macrolides, such as erythromycin. The base A2058 is involved in hydrogen bond formation with macrolide antibiotics. When A2058 is mutated to G2058, the ribosomal 23S rRNA binds the macrolide less tightly and resistance results. It turns out that the base corresponding to A2058 in eukaryotic 28S rRNA is already a G, and this difference partially explains the specificity of macrolides as bacterial translation inhibitors that are safe for use in humans.

Resistance to Tetracyclines. Besides drug modification or efflux discussed above, another clinically important type of resistance to tetracycline, called **ribosome protection**, is conferred by a cytoplasmic protein, called TetM, TetO, or TetQ in different bacteria, that protects ribosomes from tetracycline inhibition (Figure 16-6C). When the protein is present in the bacterial cytoplasm, tetracycline no longer binds to the ribosome. This mechanism does not involve covalent modification of the ribosome, which is similar to the macrolide resistance mentioned earlier.

An interesting feature of this type of resistance is that the TetO protein has GTPase activity and shares high amino acid sequence homology in its N-terminal region with bacterial elongation factor G (EF-G) involved in protein synthesis. One model, based on the crystal structure of a complex of TetO with the ribosome, is that GTP-bound TetO competes with tetracycline for binding to the ribosome. GTPase activity then converts GTP-bound TetO back to GDP-bound TetO, which is then released from the ribosome complex, allowing translation to resume. Although tetracycline efflux has been studied for decades, the ribosome protection type of resistance was discovered later and only more recently have insights into its mechanism been obtained. Nevertheless, this type of resistance appears to be quite widespread among a number of different groups of bacteria, including many Gram-positive bacteria, *Mycoplasma*, and some Gram-negative genera, such as *Neisseria, Haemophilus,* and *Bacteroides*.

A new class of tetracycline antibiotics, called glycylcyclines (such as tigecycline), was developed to circumvent this resistance mechanism and restore the effectiveness of tetracycline antibiotics. Glycylcyclines still inhibit translation by binding to the 16S rRNA, but they bind more effectively than other tetracyclines, and so are harder to dislodge from the binding site by TetO. They are also not good substrates for the tetracycline-specific efflux pumps.

Resistance to β-Lactams. Alteration of the target of the antibiotic is a second common mechanism of resistance to β-lactam antibiotics. In this case, the binding specificity of the **penicillin-binding proteins** is altered through mutation such that they no longer bind the β-lactam antibiotic. This type of resistance is particularly prevalent among Gram-positive bacteria and is currently a type of β-lactam resistance that is causing problems clinically. While β-lactamase inhibitors can counter resistance due to β-lactamases, this fix does not work for resistance due to alteration in the penicillin-binding proteins.

Probably the best-characterized resistance gene of this type is *mecA*, a gene encoding resistance to methicillin that is found in *S. aureus* (MRSA). This resistance gene encodes an alternative β-lactam-binding protein, called penicillin-binding protein 2 (PBP2a), that is not inhibited as readily by methicillin as are the normal β-lactam-binding proteins. Apparently, MecA protein replaces the normal transpeptidase and allows peptidoglycan cross-linking to occur in the presence of the β-lactam antibiotic. Expression of PBP2a is induced by β-lactam antibiotics, which is described in a subsequent section.

Another clinically important example is the development of β-lactam resistance by clinical isolates of *Streptococcus pneumoniae*. To date, genes encoding β-lactamases have not made their way to *S. pneumoniae,* and all of the observed β-lactam resistances in this bacterium are due to mutations in the chromosomal copies of the normal penicillin-binding proteins. In fact, the combinations of mutations found in the genes encoding penicillin-binding proteins in *S. pneumoniae* likely arose by HGT involving homologous recombination events during natural transformation from other *Streptococcus* species, such as the closely related *Streptococcus* *mitis* that resides in the mouths of humans. Thus, the resulting genes that impart resistance appear as "mosaics" of DNA segments that can be found in isolates of different *Streptococcus* species.

Resistance to Glycopeptide Antibiotics. Vancomycin prevents cross-linking of peptidoglycan by binding to the D-Ala-D-Ala dipeptide at the end of muropeptides. For this reason, it initially seemed that resistance to vancomycin would not readily develop because replacing the D-Ala-D-Ala dipeptide with another group that does not bind vancomycin, but still functions in cross-linking, is a very tall order. But this eventually did happen after vancomycin use as an antibiotic became widespread. **Vancomycin-resistant *Enterococcus* (VRE)** isolates, which act as opportunistic pathogens, were the first clinically important bacteria to appear that became resistant to vancomycin by replacing D-Ala-D-Ala in muropeptides with D-Ala-D-lactate, which does not bind vancomycin. **Vancomycin-resistant *S. aureus* (VRSA)** strains are now also becoming common.

There are three essential enzymes needed for this resistance phenotype (Figure 16-7). One is a ligase encoded either by *vanA* or *vanB*, which makes the D-Ala-D-lactate from D-Ala and D-lactate. A second gene, *vanH*, encodes a lactate dehydrogenase that makes D-lactate from pyruvate. These two enzymes make it possible for the bacteria to synthesize the altered part of the murodipeptide. However, as long as the bacteria still produce the original D-Ala-D-Ala, they will remain susceptible to vancomycin. This is where the third gene, *vanX*, comes into the picture. VanX is an enzyme that cleaves the D-Ala-D-Ala dipeptide precursor, but not the D-Ala-D-lactate precursor. The mechanism of vancomycin resistance is amazingly complex and shows how resourceful bacteria can be when it comes to protecting themselves from antibiotics and when the selection pressure is great enough.

Many VRE and VRSA strains have additional genes that are associated with vancomycin resistance, including *vanRS*, *vanY*, and sometimes *vanZ*. The *vanRS* operon encodes a two-component regulatory system that modulates expression of the vancomycin-resistance genes, in which VanS is the membrane-bound sensor kinase that phosphorylates the response regulator VanR, which acts as a transcriptional activator of the *vanHAX* operon. VanY is a D,D-carboxypeptidase that hydrolyzes the terminal D-Ala residue of any wild-type D-Ala-D-Ala peptidoglycan precursors that are made, which inhibits vancomycin from binding. VanZ, on the other hand, confers resistance to the related antibiotic teicoplanin by an unknown mechanism.

The origin of the vancomycin-resistance genes is still a mystery. They could have come from vancomycin-producing bacteria, such as *Amycolatopsis coloradensis*, although the currently circulating resistance genes only share about 50% to 60% amino acid identity with the corresponding genes of *A. coloradensis*. Another possible source is other Gram-positive bacteria, such as *Lactobacillus,* that are naturally vancomycin-resistant because they use D-Ala-D-Lac instead of the D-Ala-D-Ala dipeptide in their cell wall peptidoglycan. Gram-negative bacteria are also naturally resistant to vancomycin, teicoplanin, and other glycopeptides because the glycopeptides are large bulky molecules that do not diffuse readily through the outer membrane porins of Gram-negative bacteria. Intermediate resistance to vancomycin has now spread among *Staphylococcus* species, and there is considerable concern that vancomycin resistance will continue to spread to other clinically worrisome Gram-positive pathogens.

Resistance to Macrolides, Streptogramins, and Lincosamides. RNA methylases, called ErmA, ErmB, ErmF, or ErmG in different bacteria, impart simultaneous resistance to several antibiotics that bind to 23S rRNA in the ribosome. These RNA methylases add one or two methyl groups to the A2058 adenine in 23S rRNA. This is the same A base mentioned previously that can mutate spontaneously to a G base and cause resistance. The methylation of A2058 imparts widespread resistance to macrolides, streptogramins, and lincosamides, which otherwise block the ribosome exit tunnel (see chapter 15). We can surmise the mechanism for this multiple resistance from a common theme that has emerged in this chapter. The A2058 base in 23S rRNA forms important hydrogen bonds with groups in each of these antibiotic classes. Methylation of A2058 prevents hydrogen bond formation, the antibiotics fail to bind tightly to the ribosome exit channel, the tunnel remains unblocked by the antibiotic, and resistance results. This type of resistance has been found mainly in Gram-positive cocci and in *Bacteroides* species. Some Gram-negative bacteria, such as *E. coli* strains, tend to be naturally resistant to macrolides, probably because their porins do not admit the antibiotic into the periplasm.

Resistance to Quinolones, Rifampin, and Streptomycin. Resistance to quinolones commonly involves amino acid changes that alter the way these antibiotics interact with the A or B subunits of DNA gyrase. DNA gyrase is an essential enzyme, but mutations that impart resistance allow sufficient function of the gyrase to allow nearly normal growth, and eventually compensatory mutations can occur that restore full function. Similarly, resistance to rifampin is caused by mutations that result in amino acid changes in the β-subunit of RNA polymerase. These amino acid changes reduce the affinity of the antibiotic for the RNA exit channel in RNA polymerase. Finally, we have stressed the amazing interactions between rRNA and antibiotics. However, it should be kept in mind that ribosomes are complicated machines that also contain proteins. Mutations in some of these proteins can alter antibiotic binding or ribosome function in such a way that resistance results. A classic example of this form of resistance is the streptomycin resistance that results from specific amino acid changes in the S12 protein

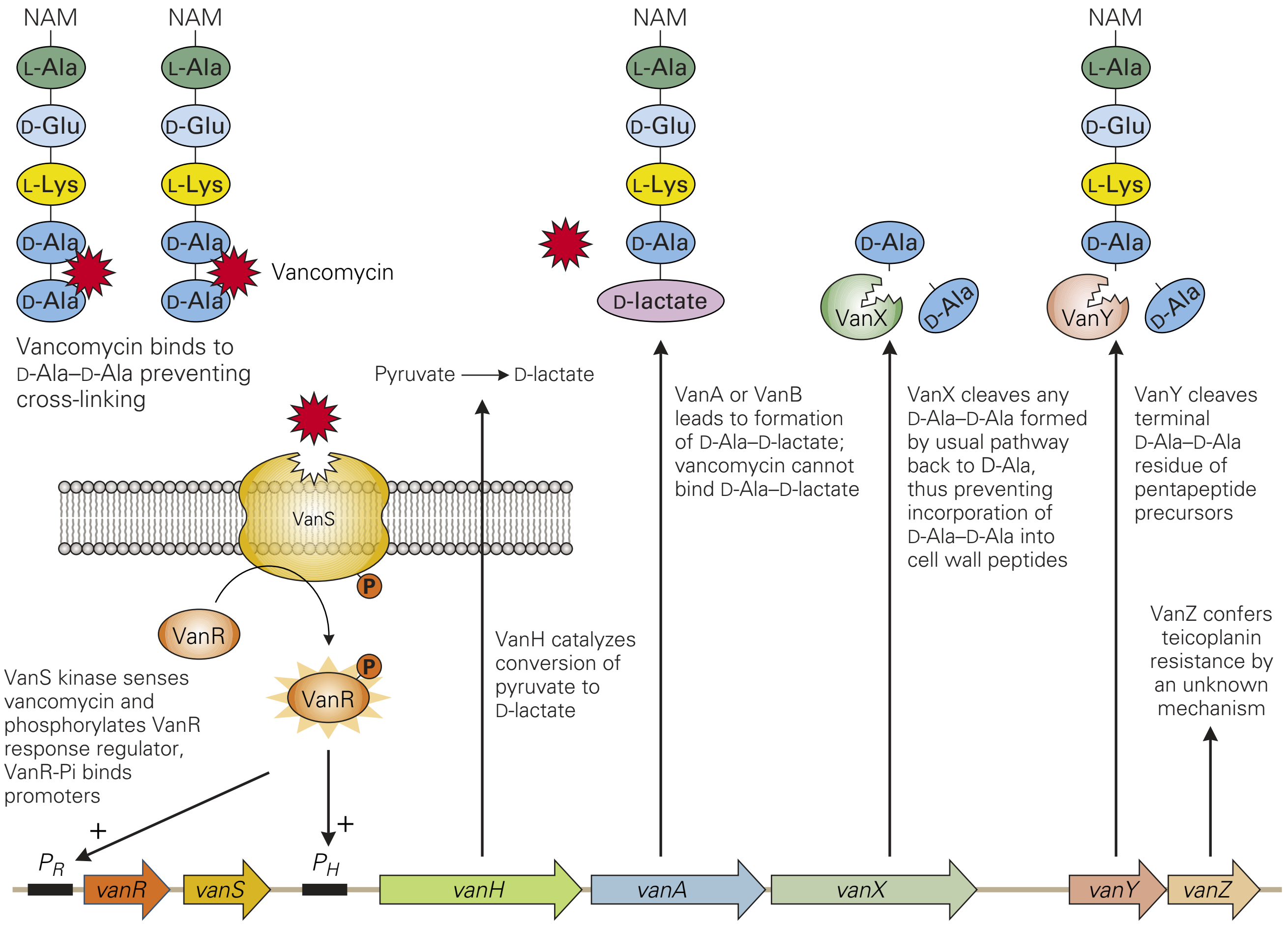
NAM
L-Ala
D-Glu
L-Lys
D-Ala
D-Ala
NAM
L-Ala
D-Glu
L-Lys
D-Ala
D-Ala
Vancomycin
Vancomycin binds to D-Ala–D-Ala preventing cross-linking
NAM
L-Ala
D-Glu
L-Lys
D-Ala
D-lactate
D-Ala
VanX
D-Ala
NAM
L-Ala
D-Glu
L-Lys
D-Ala
VanY
D-Ala
Pyruvate → D-lactate
VanS
P
VanR
P
VanR
VanS kinase senses vancomycin and phosphorylates VanR response regulator, VanR-Pi binds promoters
VanH catalyzes conversion of pyruvate to D-lactate
VanA or VanB leads to formation of D-Ala–D-lactate; vancomycin cannot bind D-Ala–D-lactate
VanX cleaves any D-Ala–D-Ala formed by usual pathway back to D-Ala, thus preventing incorporation of D-Ala–D-Ala into cell wall peptides
VanY cleaves terminal D-Ala–D-Ala residue of pentapeptide precursors
VanZ confers teicoplanin resistance by an unknown mechanism
+
+
P_R
vanR
vanS
P_H
vanH
vanA
vanX
vanY
vanZ

Figure 16-7. Mechanisms of vancomycin resistance. In susceptible bacteria, vancomycin binds to the D-Ala-D-Ala, preventing cross-linking of the peptidoglycan. Mechanisms that prevent vancomycin binding in resistant bacteria include three enzymes: VanH, which catalyzes conversion of pyruvate to D-lactate; VanA or VanB, which catalyze the synthesis of D-Ala-D-lactate; and Van X, which cleaves D-Ala-D-Ala that is synthesized by the normal pathway. Regulation of the *vanHAX* operon is controlled by the two-component system encoded by the *vanRS* operon. VanS is the sensor kinase and VanR is the response regulator. Other genes associated with VRE and VRSA strains include *vanY*, which encodes a D,D-carboxypeptidase that cleaves the terminal D-Ala residue of pentapeptide precursors with D-Ala-D-Ala residues, and *vanZ*, which confers teicoplanin resistance by an unknown mechanism. Data from Hughes D. 2003. *Nat Rev Genet* **4**:432–441.

(encoded by the *rpsL* gene) that is part of the 30S ribosomal subunit.

Resistance to Trimethoprim and Sulfonamides. Resistance to trimethoprim and sulfonamides arises from mutations in the enzymes of the folate biosynthetic pathway, which are normally competitively inhibited by these antibiotics (see Figure 15-22). The mutant forms of the enzymes no longer bind the antibiotic with a higher affinity than its natural substrate. Mutations conferring resistance to sulfonamides or trimethoprim occur rather frequently, but simultaneous double mutations that confer resistance to both types of antibiotic occur only rarely. For this reason, a combination of trimethoprim and one of the sulfonamides is currently used for antibacterial therapy.

Failure to Activate an Antibiotic

Metronidazole and Isoniazid. Metronidazole, which is often used to treat dental plaque caused by *Porphyromonas gingivalis* and gastric ulcers caused by *Helicobacter pylori*, must be activated by reduction before it can attack bacterial DNA or form thiol adducts (see Figure 15-19). Development of resistance to metronidazole by *H. pylori*, the cause of gastric ulcers, is an ominous development for ulcer sufferers. Resistance to metronidazole is poorly understood, but in at least some cases, mutations can occur that decrease the expression of the activation enzymes flavodoxin and ferredoxin that are required to convert metronidazole into its active form in the bacteria.

Isoniazid is one of the mainstays of anti-Mtb therapy. In order to work, isoniazid must first be activated by a catalase-peroxidase enzyme (KatG) produced by the mycobacteria (Figure 16-8). The activated form of isoniazid then covalently attaches to an NADH molecule to form isonicotinic acyl-NADH adduct, which binds tightly to the active site of the enoyl-acyl-carrier protein reductase, called InhA. InhA acts on long-chain fatty acids and catalyzes a step in the biosynthesis of mycolic acid, an essential part of the mycobacterial cell wall. Inhibition of InhA inhibits cell wall biosynthesis in Mtb. Known mechanisms of resistance to isoniazid in Mtb include mutations that inactivate KatG catalytic activity, prevent adduct formation, reduce the expression of the *katG* gene, or result in loss of the *katG* gene.

Regulation of Resistance Genes

Repressors. Since bacteria need resistance genes only when they encounter antibiotics, a relatively uncommon occurrence in their life, it makes sense that many antibiotic-resistance genes are regulated. The first type of regulation described for a resistance mechanism was repression of the genes encoding tetracycline efflux pumps. In *E. coli*, the amount of the TetA pump is regulated by classical repression control mediated by the TetR repressor (Figure 16-9A). When tetracycline is absent from the cytoplasm, TetR binds to an operator that blocks high levels of transcription of the *tetA* gene. The *tetR* gene, encoding TetR, is divergently transcribed from *tetA,* and *tetR* transcription is autoregulated by this repression mechanism. When tetracycline is present, it enters the cell, complexes with Mg^{2+} ion, and then binds to TetR, which causes release of TetR from the operator DNA, thereby allowing high-level expression of the TetA pump.

Repression mechanisms regulate many other resistance genes. In MRSA, there is an interesting variation of the repression mechanism (Figure 16-9B). In the absence of β-lactam antibiotics, a repressor called BlaI inhibits the transcription of the *blaZ* gene, which encodes a β-lactamase. BlaI also autoregulates its own transcription by turning off the *blaI* gene and other genes in the signal transduction pathway. When cells encounter β-lactam antibiotics, a serine residue in a surface protein called BlaR1 forms a covalent bond with the β-lactam ring analogous to the one that forms in transpeptidases or serine β-lactamases (see above). This binding signals the BlaR1 protein, which extends all the way through the membrane, to release a dormant protease called BlaR2 into the cytoplasm. The release activates BlaR2 for cleavage of its substrate, BlaI. As the intact BlaI repressor disappears, the *blaZ* gene is transcribed, which allows for production of the BlaZ β-lactamase. BlaZ is exported to the cell surface, where it inactivates the β-lactam antibiotic. This multiple-component repression system is much more elaborate than the classical repression of expression of the TetB efflux pump, where tetracycline itself relieves the repression directly. Nevertheless, it accomplishes a similar goal and allows MRSA to express the BlaZ β-lactamase only when it needs it. A similar protease-dependent repression system regulates expression of the alternate PBP2a protein by MRSA, which is more resistant to β-lactam antibiotics than the ones expressed in the absence of antibiotic (see earlier section).

Translational Attenuation. Another type of regulation of resistance genes, first described for ***erm*** RNA methylase genes of Gram-positive bacteria, is a form of **translational attenuation** (Figure 16-10A). The mRNA for the resistance gene starts nearly 100 bp upstream of the start codon for the gene. This 100-bp **leader region** in the transcript encodes a short peptide (**leader peptide**). In the absence of the macrolide, ribosomes move rapidly along the mRNA and the leader peptide is efficiently translated. Under these conditions, two **stem-loop structures** form in the mRNA in such a way that the second stem-loop structure masks the ribosome-binding site and start codon of the *erm* gene, thereby preventing translation of the *erm* gene product,

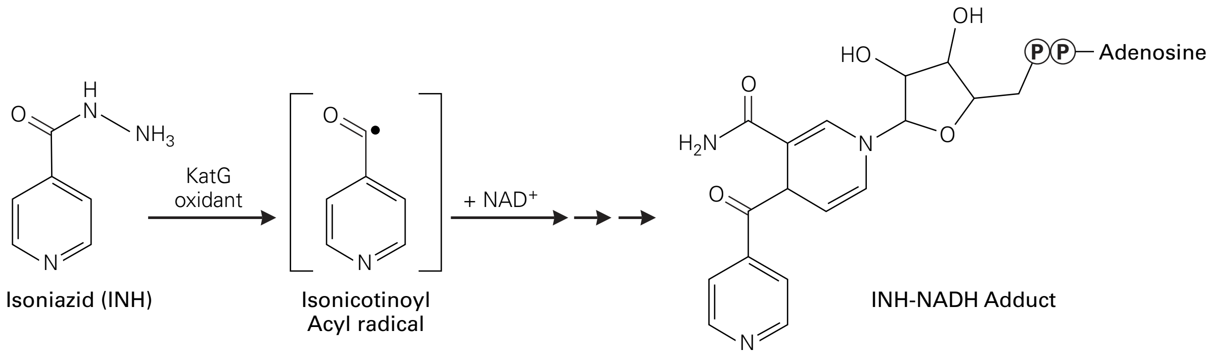

Figure 16-8. KatG catalyzed activation of isoniazid and inhibition of mycolic acid biosynthesis. KatG catalyzes activation of isoniazid in the presence of an oxidant, such as hydrogen peroxide (H_2O_2). The isonicotinoyl radical forms an adduct with NADH that binds tightly to InhA, an enoyl-acyl-carrier protein reductase involved in the biosynthesis of mycolic acid, a component of the mycobacterial cell wall.

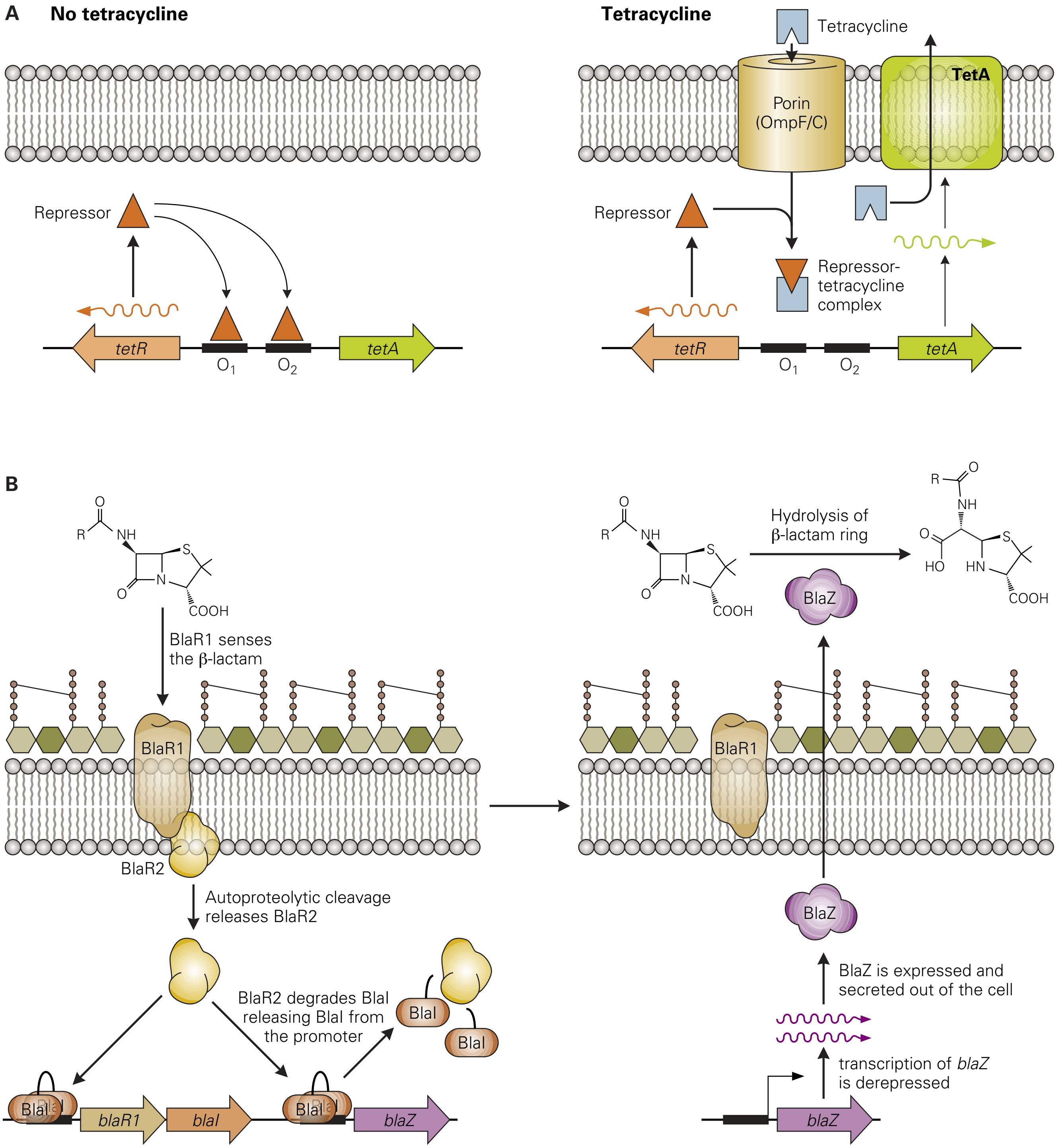
A
No tetracycline
Repressor
tetR
O1
O2
tetA
Tetracycline
Tetracycline
Porin (OmpF/C)
TetA
Repressor
Repressor-tetracycline complex
tetR
O1
O2
tetA
B
R
O
NH
S
N
O
COOH
BlaR1 senses the β-lactam
BlaR1
BlaR2
Autoproteolytic cleavage releases BlaR2
BlaR2 degrades BlaI releasing BlaI from the promoter
BlaI
BlaI
BlaI
BlaI
blaR1
blaI
blaZ
Hydrolysis of β-lactam ring
BlaZ
HO
HN
COOH
BlaR1
BlaZ
BlaZ is expressed and secreted out of the cell
transcription of blaZ is derepressed
blaZ

Figure 16-9. Repressor-mediated regulation of antibiotic-resistance genes. (A) Tetracycline (Tc) and TetR regulation of the tetracycline efflux pump gene *tetA* in *E. coli*. Tc enters the periplasm as a complex with Mg^{2+} through porins, such as OmpF/C, where it can dissociate from Mg^{2+} and cross the inner membrane through diffusion. In the cytoplasm, Tc complexes with Mg^{2+} again and binds to the TetR repressor that is bound to the *tet* operators O_1 and O_2 located in the promoter region of the operon. The Tc-TetR complex dissociates from the operators, allowing for transcription of the *tetR* and *tetA* genes. The H^+-dependent TetA antiporter then exports the intracellular Tc-Mg^{2+} complexes out of the cell. **(B)** Regulation of the BlaZ β-lactamase gene in MRSA. In the absence of β-lactam antibiotic, BlaI represses the *blaZ-blaR1-blaI* genes. When BlaR1 senses the presence of antibiotic, the cytoplasmic domain of BlaR1 is autoproteolytically cleaved. The released BlaR2 proceeds to proteolytically cleave the repressor BlaI, which enables transcription of the β-lactamase gene *blaZ*. BlaZ is expressed and secreted, leading to hydrolysis of the β-lactam antibiotic.

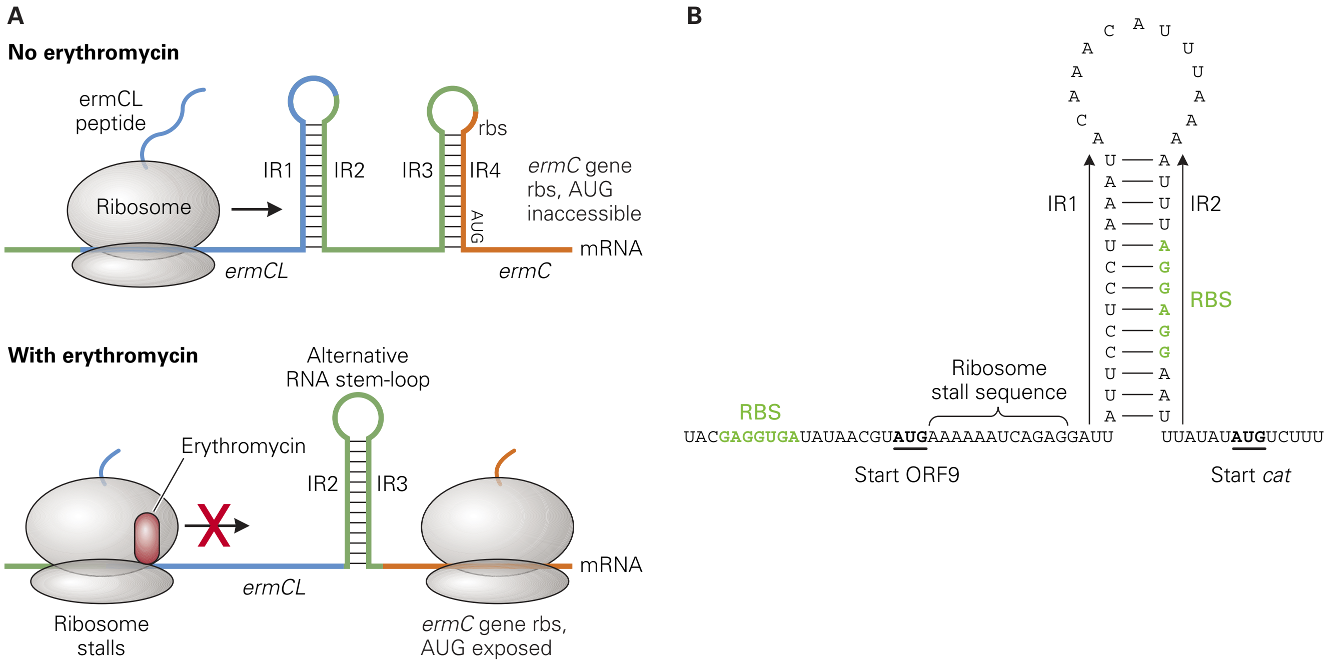

Figure 16-10. **Regulation of antibiotic-resistance genes by translational attenuation. (A)** Mechanism of translation attenuation regulating erythromycin-induced expression of the *ermC* resistance gene. In the absence of erythromycin, there are two stem loops formed from the inverted repeats, 1+2 and 3+4, and the ribosome binds the first RBS and translates the upstream regulatory ORF, *ermCL*, but does not bind the second RBS, which is occluded by the stem loop and does not translate the *ermC* mRNA. Erythromycin binds to the ribosome and causes the ribosome to stall, which causes the formation of a stem loop between the inverted repeats, 2+3. This exposes the second RBS, allowing another ribosome to bind and translate the *ermC* mRNA. **(B)** Mechanism of translation attenuation regulating chloramphenicol-induced expression of the *cat* mRNA. In this case, a single stem-loop structure is present in the absence of chloramphenicol, which blocks ribosome binding to the second RBS directly upstream of the *cat* gene. Binding of chloramphenicol causes the stalled ribosome to change conformation and open the stem loop, which allows another ribosome to bind and translate the *cat* mRNA.

RNA methylase. When erythromycin is present, the bacterial ribosomes cannot translocate and thus do not move along the mRNA. Stalling during translation of the *erm* leader peptide allows formation of an alternative RNA stem-loop structure so that the ribosome-binding site and start codon are now exposed, allowing *erm* gene translation and resistance. Methylase-modified ribosomes do not stall during translation of the *erm* leader peptide, allowing autoregulation to reduce *erm* gene translation according to need. Conversely, low-level formation of the alternative stem-loop structure in the absence of macrolides allows translation of basal amounts of the methylase to be synthesized to keep some ribosomes functional when the antibiotic is again encountered.

A similar translational attenuation regulation occurs with some chloramphenicol resistance *cat* genes, whose expression is induced by the antibiotic (Figure 16-10B). In this case, there are two ribosomal binding sites (RBS) and translational AUG start sites associated with the *cat* gene, one encoding a short peptide ORF9 and the other encoding the actual *cat* gene. In between the two start sites is a pair of inverted repeats, IR1 and IR2, that form a stem-loop structure in the mRNA that occludes one of the RBS regions. The ORF9 mRNA contains a ribosomal stall sequence that is complementary to the 3'-end of the 16S rRNA. When the ribosome binds and then stalls at this position it opens the stem loop, which in turn exposes the second RBS immediately upstream of the *cat* gene. A second ribosome can then bind to this RBS and translate the *cat* mRNA. Binding of the chloramphenicol to the ribosome triggers conformational changes that allow base pairing of the 3'-end of the 16S rRNA with the complementary stall sequence, thereby mediating induction of *cat* gene expression and antibiotic resistance.

Activators. Many resistance genes are regulated by **transcriptional activators.** In this mechanism, the antibiotic generates a signal molecule that binds to the activator protein. This complex then binds to the promoter region of the resistance gene and increases transcription of the gene. An excellent example of this mechanism is activation of the vancomycin-resistance genes from *Enterococcus* species by the VanRS two-component regulatory system (see Figure 16-7). The VanS histidine kinase senses cell wall damage caused by vancomycin. The signal sensed by the VanS extracellular domain may be a cell wall fragment, but does not seem to be vancomycin itself. Binding of this signal is transduced by VanS across the cell membrane and leads to autophosphorylation of a specific histidine residue in a cytoplasmic domain of VanS. This phosphoryl group is then transferred to a specific aspartate residue in the VanR response regulator, which alters its conformation to an activated state. Phosphorylated VanR then binds to the promoter regions upstream of the *vanRS* operon (autoregulation) and the *vanHAX* operons (encoding the muropeptide modification enzymes described earlier) and activates their transcription.

A different mechanism regulates activation of expression of the *ampC*-encoded β-lactamase (AmpC) in *E. coli, P. aeruginosa,* and some other Gram-negative bacteria (Figure 16-11). Inhibition of transpeptidation by β-lactam antibiotics induces cell wall autolysis that leads to accumulation of peptidoglycan fragments, including 1,6-anhydrodisaccharide tripeptide (box in Figure 16-11). This peptidoglycan fragment is transported into the cytoplasm by the AmpG protein, where it is processed for recycling by NagZ, which removes the N-acetylglucosamine sugar to release 1,6-anhydromuramyl tripeptide. AmpD amidase then removes the sugar from the tripeptide, which is recycled to form the cell wall precursor, UDP-NAM-pentapeptide. This inhibitory signaling molecule binds to the AmpR protein, which then changes conformation, and the complex binds upstream of the *ampC* gene and allows for only basal levels of transcription. The AmpC β-lactamase is exported to the periplasm of the cell, where it degrades the β-lactam antibiotic. This system is fine-tuned by the AmpD amidase protein, which ensures that high concentrations of the anhydromuramyl tripeptide signal will accumulate only when there is significant damage to the cell wall in response to β-lactam antibiotics. However, in the presence of increased β-lactam antibiotics, AmpD cannot process all of the 1,6-anhydromuropeptides that enter the cell, and so these 1,6-anhydromuropeptides compete for binding to the AmpR, instead of the inhibitory UDP-NAM-pentapeptides, and cause AmpR to act as a transcriptional activator of *ampC*, which increases AmpC production and results in enhanced β-lactam antibiotic resistance. Mutations that inactivate AmpD or reduce expression of AmpD lead to enhanced activation of AmpR-mediated transcription of *ampC* and increased AmpC production and β-lactam antibiotic resistance.

Insertion Sequences and Promoter Mutations. Although transcriptional regulation mechanisms are usually reversible, so that expression of a gene can be turned off as well as on, there is a type of increased transcription that might be considered regulatory even though it results in a permanent alteration. Mutations in a promoter region or insertions of a transposon upstream of a resistance gene can increase the expression of the resistance gene and thus the level of resistance. For example, a noninducible version of the *ampC* gene in *Enterobacter* was originally expressed at such a low level that it did not make the bacteria resistant to ampicillin. Gradually, with continued selection by antibiotic use, mutations accumulated in the promoter region that increased β-lactamase gene expression to the point that it has become a serious contributor to antibiotic resistance. In this case, transcription by the mutant promoter is constitutively on, and the β-lactamase enzyme is always produced.

Other resistance genes that were originally silent when they entered a new host could later acquire an insertion sequence in their promoter region that would cause the gene to be expressed. Many insertion sequences have promoters that point outward from their ends. Thus, when they insert into a region upstream of an open reading frame, they provide a promoter that now controls expression of the gene. An example of this is a plasmid-borne erythromycin-resistance gene in *Bacteroides* species, *ermF*, which is expressed because it has acquired a promoter from an adjacent insertion sequence. Because there are many such examples of promoter mutations or insertion of an insertion sequence that activate the expression of a gene, scientists now consider any resistance gene that enters a bacterial strain, whether it is expressed initially or not, to be of concern because it can be activated through mutation over time.

Antibiotic Tolerance and Persister Cells

Antibiotic Tolerance

The antibiotics that inhibit cell wall synthesis are bactericidal because the bacterium participates in its own destruction; bacterial enzymes that normally participate in cell wall turnover (lytic enzymes or autolysins) degrade the peptidoglycan, leaving the bacterium without the protection of its cell wall. Bacteria that can prevent their autolysins from destroying their peptidoglycan, that are not actively growing, or that are located in an environment where they can survive without a cell wall can avoid being killed by the antibiotic. This type of response to antibiotics is called **tolerance**. Tolerance differs from resistance. A resistant bacterium continues to grow in the presence of the antibiotic. In contrast, a tolerant bacterium stops growing when the antibiotic is present but is not killed. When the antibiotic levels fall, tolerant bacteria have a chance to recover and resume growth. Unlike resistance, tolerance is not due to mutations or acquisition of additional genes and is reversible.

A different type of tolerance has been seen in the case of *E. coli* strains that cause kidney infections. Because the kidney filters blood and excretes wastes in urine, the concentration of salts in the kidney is higher than in other tissues. Thus, when *E. coli* loses its cell wall because penicillin stops cell

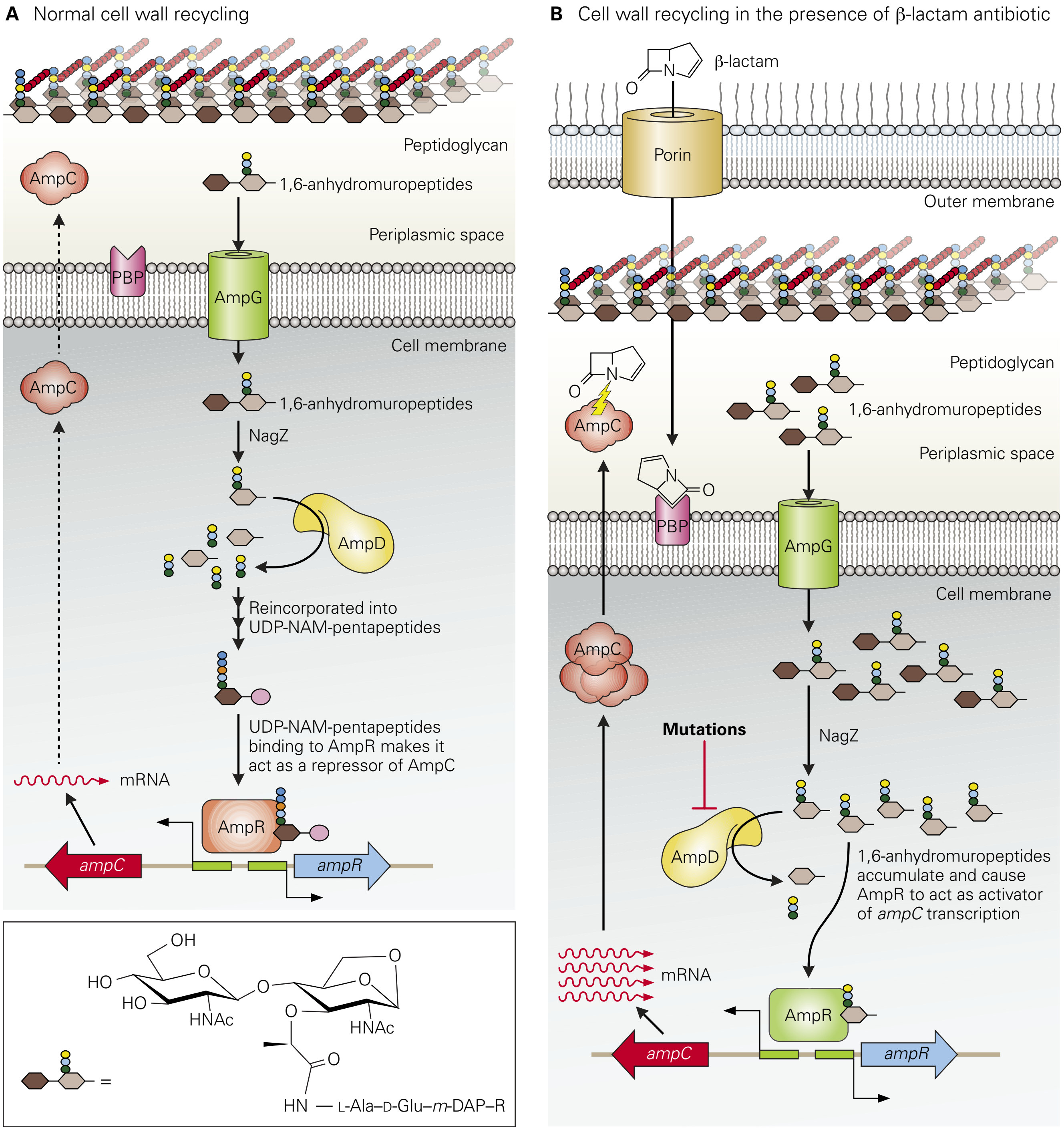
A Normal cell wall recycling
Peptidoglycan
AmpC
1,6-anhydromuropeptides
Periplasmic space
PBP
AmpG
Cell membrane
AmpC
1,6-anhydromuropeptides
NagZ
AmpD
Reincorporated into
UDP-NAM-pentapeptides
UDP-NAM-pentapeptides
binding to AmpR makes it
act as a repressor of AmpC
mRNA
AmpR
ampC
ampR
OH
HO
O
O
O
O
HO
HNAc
HNAc
O
O
=
HN — L-Ala–D-Glu–m-DAP–R
B Cell wall recycling in the presence of β-lactam antibiotic
O
N
β-lactam
Porin
Outer membrane
Peptidoglycan
O
N
AmpC
1,6-anhydromuropeptides
N
O
Periplasmic space
PBP
AmpG
Cell membrane
AmpC
Mutations
NagZ
AmpD
1,6-anhydromuropeptides
accumulate and cause
AmpR to act as activator
of ampC transcription
mRNA
AmpR
ampC
ampR

Figure 16-11. **Transcriptional regulation of AmpC β-lactam antibiotic-resistance gene expression by AmpR and AmpD.** In *E. coli* and *P. aeruginosa*, the transcriptional activator AmpR and the amidase AmpD regulate AmpC-mediated β-lactam antibiotic resistance. **(A)** During normal cell wall recycling, 1,6-anhydromuropeptides are removed from the cell wall, transported back into the cell by the AmpG permease, and hydrolyzed by the glycoside hydrolase NagZ, which removes the N-acetylmuramyl (NAM) group, and by AmpD to generate free tripeptides, which are then recycled back into UDP-NAM-pentapeptide cell wall precursors. This inhibitory signaling molecule binds to the transcriptional regulator AmpR, which is bound to the promoter region of the *ampC* gene and acts as a transcriptional repressor, allowing only basal levels of *ampC* expression. The AmpC is exported to the periplasm. **(B)** The β-lactam antibiotic enters through the outer membrane porin and binds to penicillin-binding proteins (PBPs). This causes increased accumulation of 1,6-anhydromuropeptides, which are transported into the cytoplasm. AmpD cannot process all of the 1,6-anhydromuropeptides, which act as activating signaling molecules that compete with the UDP-NAM-pentapeptides for binding to AmpR, converting the AmpR into a transcriptional activator that increases transcription of *ampC* to produce more AmpC β-lactamase. Mutations that inactivate AmpD or reduce expression of AmpD lead to increased AmpC production and β-lactam antibiotic resistance. Adapted from Lister PD, Wolter DJ, Hanson ND. 2009. *Clin Microbiol Rev* **22**:582–610, and Walsh C. 2003. *Antibiotics: Actions, Origins, Resistance.* ASM Press, Washington, DC, with permission.

wall synthesis and autolysins degrade the existing cell wall, the osmotic strength of the fluid in which the bacteria are bathed is high enough to keep the bacteria from lysing due to internal turgor pressure. These forms of *E. coli* and other bacteria that lack a cell wall have been called L forms.

Persistence

Tolerance is particularly significant in the case of bacterial biofilms, which are associated with the majority of infections involving catheters, orthopedic devices, heart valves, the urinary tract, and the lungs of cystic fibrosis patients. Interestingly, it has been found that a small percentage (about 1%) of dormant bacterial cells within a biofilm, called **persister cells**, contribute to the high level of tolerance observed in the bacterial population. These persister cells appear to be nongrowing bacterial cells in a state of **dormancy**, defined as metabolically inactive but still viable, that can survive antibiotic treatment and repopulate (Figure 16-12). It is believed that persister cells contribute to the high level of tolerance observed in biofilms. The molecular basis for the presence of persister cells and how they lead to tolerance is not well understood. It seems likely that there may be multiple mechanisms that can cause persisters to appear at different stages of growth.

Recent data has determined that bacterial populations are not uniform with respect to the expression of certain genes. Noise in regulatory circuits, formally called **stochastic processes,** can lead to the appearance of subpopulations with different phenotypes and environmental responses. One idea is that persisters represent just such a subpopulation in which certain metabolic stress genes are induced that inhibit macromolecular synthesis processes susceptible to antibiotics, such as translation and cell wall biosynthesis. In some instances, certain inhibitory signal molecules, such as the **alarmone (p)ppGpp**, shut down the growth of the persister subpopulation and allow it to weather the presence of antibiotics.

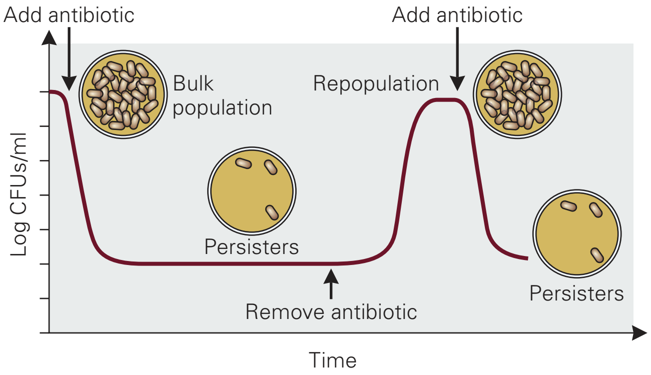

Figure 16-12. Bacterial persistence. The bulk of a population of antibiotic-sensitive bacteria growing in culture is efficiently killed by addition of antibiotic. Beyond a certain threshold, a plateau is reached, where a small percentage of cells (1% of the initial population) remain viable. These persister cells can repopulate when the antibiotic is removed, but are still sensitive to antibiotic. Adapted from Verstraeten N, Knapen W, Fauvart M, Michiels J. 2016. *Methods Mol Biol* **1333:**3–13, with permission.

Toxin-Antitoxin Systems

Over the past decade, additional metabolic stress genes have been identified. In particular, two-gene operons, encoding so-called **toxin-antitoxin (TA) systems**, have been demonstrated to serve as key regulators of the persister state. TA systems are comprised of a toxin, which induces growth arrest by blocking an essential cellular function, and its cognate antitoxin, which acts as a bacterial "immunity" protein that binds tightly to the toxin and neutralizes its toxic activity. The toxin is typically a protein or peptide, while the antitoxin can be a regulatory sRNA or a protein. (Note that these TA systems are distinct from other types of toxins that we discussed in chapter 12.)

TA systems were first shown to be important for plasmid maintenance, in which bacterial progeny were killed that did not retain the plasmid encoding the TA system because they still had the stable toxin present but lacked the less stable antitoxin, which was no longer being replenished. Since their first discovery, many different types of TA systems have been identified, encoded by plasmids, bacteriophages, or chromosomes. Unlike the plasmid-borne TA systems that function primarily to maintain plasmids, TA systems found on chromosomes serve as important survival strategies for bacterial populations under stress conditions.

There are six known classes of TA systems (types I through VI) that are categorized according to the mechanism used by the antitoxin to neutralize the toxin activity (Figure 16-13).

- Type I: The toxin encodes a membrane-disrupting peptide that lyses the cell. The antitoxin is an sRNA that base pairs with the mRNA of the toxin to inhibit its translation.
- Type II: Both the toxin and antitoxin are proteins that under normal growth conditions form tightly bound complexes. Under stress conditions, bacterial proteases, such as Lon or ClpXP, are activated and cleave the antitoxin, releasing the toxin, which then inhibits cell growth.
- Type III: The toxin is an RNase that processes the sRNA of the antitoxin to form a pseudoknot-toxin complex that blocks toxin activity.
- Type IV: The protein antitoxin competes with the toxin to stabilize bacterial filaments. In its absence, the protein toxin destabilizes the filaments and inhibits cell division.
- Type V: The toxin encodes a membrane-disrupting peptide that lyses the cell. The antitoxin is an RNase that specifically processes the mRNA of the toxin. Under stress conditions, the mRNA of the antitoxin is degraded by another RNase, which results in translation of the toxin and toxin-peptide-mediated membrane lysis.

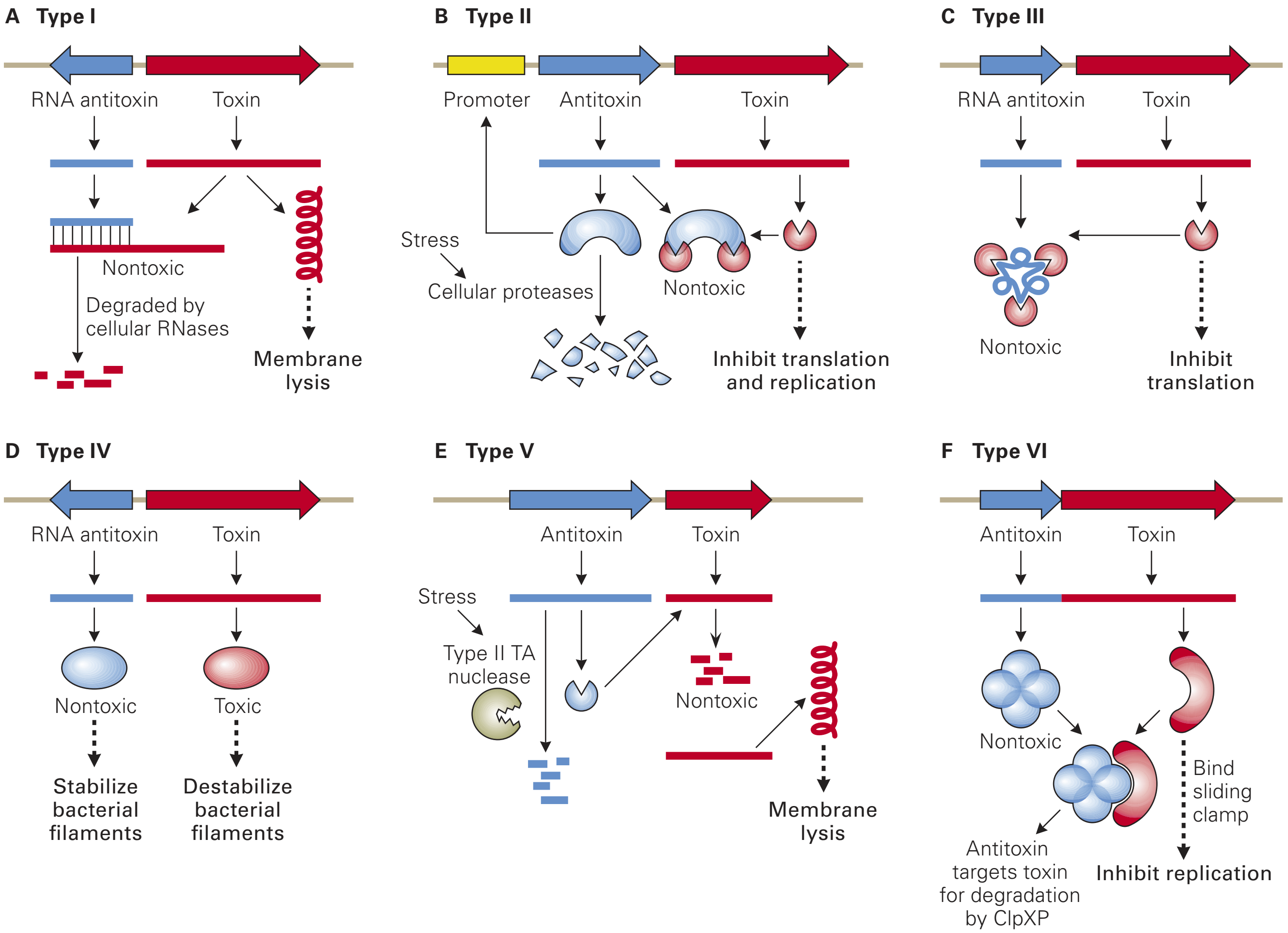
A Type I
RNA antitoxin
Toxin
Nontoxic
Degraded by cellular RNases
Membrane lysis
B Type II
Promoter
Antitoxin
Toxin
Stress
Cellular proteases
Nontoxic
Inhibit translation and replication
C Type III
RNA antitoxin
Toxin
Nontoxic
Inhibit translation
D Type IV
RNA antitoxin
Toxin
Nontoxic
Toxic
Stabilize bacterial filaments
Destabilize bacterial filaments
E Type V
Antitoxin
Toxin
Stress
Type II TA nuclease
Nontoxic
Membrane lysis
F Type VI
Antitoxin
Toxin
Nontoxic
Bind sliding clamp
Antitoxin targets toxin for degradation by ClpXP
Inhibit replication

Figure 16-13. Classes of bacterial toxin-antitoxin systems. Shown are the six classes of toxin-antitoxin (TA) systems, types I through VI. Toxins are colored red and antitoxins blue. In type I TA systems, the sRNA antitoxin base pairs with the toxin mRNA to inhibit translation of the toxin, which if not inhibited would insert into membranes and cause cell lysis. In type II TA systems, both toxin and antitoxin are proteins. The antitoxin binds tightly to the toxin to act as transcriptional repressor of the TA operon. Under stress conditions, cellular proteases (e.g., Lon and ClpXP) cleave the antitoxin to release the toxin, which then inhibits translation and replication so the bacteria do not grow. In type III TA systems, the antitoxin sRNA is processed by the RNase toxin to form toxin-pseudoknot complexes that sequester the toxin from inhibiting translation. In type IV TA systems, the antitoxin protein stabilizes bacterial filaments, while the toxin protein destabilizes them to cause inhibition of cell division. In type V TA systems, the antitoxin GhoS is an RNase specific for the toxin *ghoT* mRNA. Under stress conditions, the antitoxin *ghoS* mRNA is degraded by the MqsR RNase, which protects the *ghoT* mRNA to allow translation. The resulting GhoT toxin is an amphipathic helical peptide that causes membrane lysis. In type VI TA systems, the antitoxin SocA is an adaptor protein that binds the toxin SocB to promote its degradation by the cellular protease ClpXP. In the absence of SocA, SocB binds to the sliding clamp to inhibit DNA replication. Adapted from Page R, Peti W. 2016. *Nat Chem Biol* **12**:208–214, with permission.

- Type VI: The toxin protein binds to the sliding clamp of the DNA replisome and inhibits DNA replication. The antitoxin is an adaptor protein that binds to the toxin to promote its degradation by the protease ClpXP.

TA systems may play roles in the persistence of certain bacterial pathogens, such as Mtb. Evidence points to a role for activation of the host-acquired immune response in mediating antibiotic tolerance and the persister state in Mtb. Transcriptional profiling of Mtb-infected macrophages with or without drug treatment during infection revealed that drug treatment and host-cell activation both lead to upregulation of bacterial stress regulons, primarily those related to reactive oxygen and nitrogen species, common to four different frontline drugs: isoniazid, rifampin, ethambutol, and pyrazinamide. While the overall bacterial burden is reduced upon activation of macrophages and DCs, those bacteria that do survive become stress-adapted, more metabolically quiescent, slower-replicating (dormant), and consequently more refractory to killing by antibiotics. Mtb strains have a large number of TA systems, some of which have recently been shown to contribute to the transition into the dormant or persister state.

Horizontal Gene Transfer (HGT) of Resistance Genes

Bacteria can become resistant to antibiotics through the mutation of existing genes, but this process is very expensive for the bacteria in terms of fitness and survival. In the process of testing all the different mutations possible for any given gene essential for survival, many bacteria will die. Sometimes a single mutation is sufficient to confer resistance. This is the case with fluoroquinolone resistance. Single mutations in the bacterial DNA gyrase can make the enzyme unable to bind or respond to the antibiotic, thereby conferring resistance to the fluoroquinolone on the bacteria. In such cases, mutation to resistance is a feasible option for the bacterium. By analogy, bacteria readily accumulate spontaneous mutations in the gene encoding the β-subunit of RNA polymerase that confers resistance to rifampin. However, in other cases, such as resistance to penicillins or tetracyclines, several mutations are needed, and the development of resistance by accumulation of spontaneous mutations can take a long time, usually requiring repeated selection at sublethal concentrations of the antibiotic. In some cases, such as resistance to vancomycin, resistance does not develop by spontaneous mutation.

A much easier, quicker, and safer way for a bacterium to become resistant to an antibiotic is to acquire the resistance gene or genes from some other bacterium through horizontal gene transfer (HGT, which is covered in detail in chapter 7). The potential power of this rapid mechanism for evolution of resistance has been demonstrated in a number of studies, which showed that *Salmonella* and *E. coli* can invade and be taken up by plants, such as lettuce, and that the bacteria can exchange DNA, not only on the plant surface but also within the plant cells. Couple this with the knowledge that genetic exchange among bacteria is frequent within animal guts and within insect midguts, and could even occur within animal epithelial cells, and the implications of these findings are far-reaching and quite alarming. If water contaminated with bacteria containing antibiotic-resistance genes is used for irrigation of crops, it is possible that resistance could spread to other bacteria. Moreover, if animals or humans then eat the crops, those bacteria present in the food could exchange DNA with our resident gut microbiota and confer resistance to them and subsequently any potential pathogens that might come along.

Scientists believe that the most common mode of acquiring resistance genes, especially when the donor is a member of a different species, is by **conjugation**. Although bacteria can acquire new genes by bacteriophage **transduction** or by **transformation** (uptake of DNA from the external environment), these types of transfer tend to occur mainly between members of the same species or members of closely related species. The reason is that in phage transduction, the bacteria have to have the right phage receptor on their surfaces, a trait that is usually restricted to a closely related group of bacteria. In the case of natural transformation, in which linear single-stranded fragments of DNA are taken up by a specialized set of proteins, the DNA must integrate into the genome by homologous recombination. Thus, bacteria must be close enough to each other genetically for homologous recombination to be possible. Although conjugation also has similar limits with regard to host range (requiring particular receptors or specific uptake recognition sequences in the DNA) and compatibility (defined by toxin-antitoxin systems), conjugation can circumvent many of these limitations.

Narrow-host-range resistance gene transfer can be important clinically. For example, transformation may be transferring the mutant penicillin-binding proteins that make *S. pneumoniae* resistant to penicillin. However, the spread of resistance genes, especially between members of different species, can be a much more serious general threat. Accordingly, attention has tended to focus on the transfer of resistance genes by conjugation. Conjugation is the direct cell-to-cell transfer of DNA through a protein complex that transits the membranes of two bacteria. As detailed in chapter 7, there are two types of conjugative elements: plasmids and conjugative transposons.

The best-studied type of conjugative element is the conjugative plasmid (Figure 16-14). Not all plasmids are capable of self-transfer. Plasmids that transfer themselves by conjugation must carry a number of genes encoding proteins needed for the conjugation process itself (*tra* genes). Thus, **self-transmissible plasmids** are usually at least 25 kbp in size. Some plasmids that cannot transfer themselves can still be mobilized by self-transmissible plasmids. These are called **mobilizable plasmids** and can be much smaller than self-transmissible plasmids because they need only one or two genes (***mob* genes**) to take advantage of the transfer machinery provided by the other plasmid.

Clinical isolates resistant to many different types of antibiotics are being seen with increasing frequency. This multidrug resistance can arise in two ways. First, some types of resistance mechanisms, such as mutations in Gram-negative porins or some types of antibiotic efflux, confer resistance to more than one type of antibiotic. Second, mobile genetic elements, such as plasmids, can acquire multiple resistance genes. Thus, acquisition of a single plasmid can make the recipient resistant to multiple drugs. As discussed in chapter 7, multiple virulence determinants can also be transferred by plasmids, often on the same plasmid as the resistance genes. Many examples of plasmids carrying multiple resistance genes have been reported, but until recently it was not clear how such plasmids arose.

A plasmid can pick up more than one resistance gene if the resistance genes are carried on **transposons**, DNA segments that can insert into a chromosome or plasmid independently of homologous recombination. Transposons are flanked by DNA segments known as **insertion sequence (IS) elements**, which encode the enzyme that catalyzes

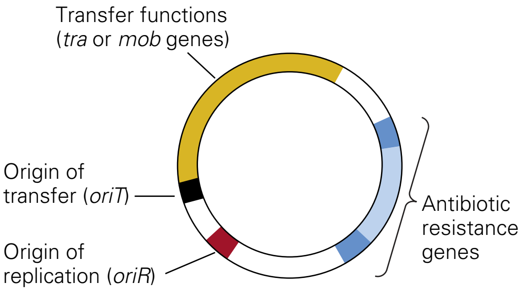

Figure 16-14. Characteristics of conjugative plasmids carrying antibiotic-resistance genes. Conjugative plasmids have both an origin of replication (*oriR*), which allows for autonomous replication in the bacteria, and an origin of transfer (*oriT*), which allows for self-transfer of the plasmid from the donor bacterial cell to a recipient cell. Conjugative plasmids also contain a set of transfer (*tra*) genes that mediate the conjugative transfer between the donor and recipient cells via the sex pili encoded by some of the *tra* genes. Some plasmids are mobilizable with the assistance of other fully conjugative plasmids that carry *tra* genes, since they have only a subset of the genes required for transfer, called the mobilization (*mob*) genes. The proteins encoded by the *mob* genes nick the plasmid DNA at *oriT* and initiate transfer of the ssDNA into the recipient cell using the conjugative system encoded by the *tra* genes.

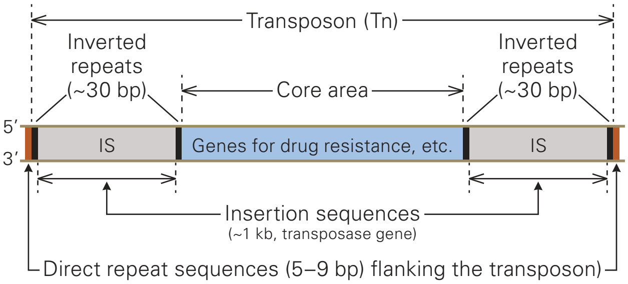
Transposon (Tn)
Inverted
repeats
(~30 bp)
Core area
Inverted
repeats
(~30 bp)
5′
3′
IS
Genes for drug resistance, etc.
IS
3′
5′
Insertion sequences
(~1 kb, transposase gene)
Direct repeat sequences (5–9 bp) flanking the transposon)

Figure 16-15. Basic structure of a transposon. A transposon is composed of two direct-repeat insertions sequence (IS) elements and intervening DNA, which can carry antibiotic-resistance genes or genes conferring other traits, such as toxins or capsule biosynthetic enzymes. The IS elements and the DNA they flank move as a single unit among bacteria.

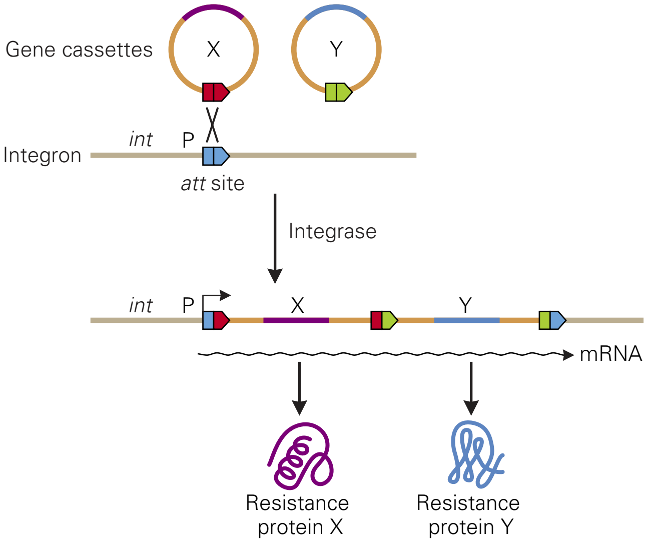

Figure 16-16. Integration of two gene cassettes, carrying promoterless resistance genes X and Y, into an integron. The integron supplies the promoter (P) and an integrase gene (*int*). The gene cassettes, each carrying genes for antibiotic resistance (or other functions), undergo site-specific and direction-specific insertion into the integron *att* site. Although two genes X and Y are shown here, some integrons can accumulate many genes.

transposition (**transposase**) and provide the ends recognized by transposase when it cuts and pastes the DNA during an insertion event (Figure 16-15). Different IS elements have sequence recognition sites (motifs) that make it possible to recognize them from their DNA sequences, even when transposition activity cannot be observed. Between the IS elements are resistance genes or other genes not involved in transposition, which are also moved with the transposon. Some multiresistance plasmids may have arisen by acquiring sequential transposon insertions.

Another type of integrating element, called an **integron**, is probably responsible for the evolution of many of the plasmids that carry multiple resistance genes. Integrons are usually transposons, but they have an extra feature. They contain a gene that encodes a **site-specific recombinase (integrase)** and an **attachment (*att*) site** that is recognized by the integrase for insertion of gene cassettes. The integrase integrates circular DNA segments containing a promoterless open reading frame (gene cassette) into the *att* site (Figure 16-16). In effect, integrons create operons by sequential integration of the gene cassettes. The *att* site has a promoter *P* provided by the integron that directs transcription of the cassette-encoded operon genes.

A second type of conjugative element is the **conjugative transposon**. Conjugative transposons are usually located in the bacterial chromosome and can transfer themselves from the chromosome of one bacterial cell (called the donor) to another bacterial cell (called the recipient). Once in the recipient, the transposon can then integrate into the recipient's chromosome and any plasmids that may be present. There are many different mechanisms of gene transfer among elements found in transposons, phages, and plasmids. Some transposons transfer via duplication. Others excise themselves from the donor genome by a process referred to as precise or nearly precise excision, whereby the excision occurs between two short direct-repeat sequences that results in deletion of all the intervening sequences plus one of the direct repeats (Figure 16-17). In these cases, the transfer intermediate is a covalently closed circle that does not replicate, but transfers similarly to a plasmid. In the recipient, they integrate into the chromosome by a mechanism that does not duplicate the target site.

Conjugative transposons are probably responsible for at least as much resistance gene transfer as plasmids, especially among Gram-positive bacteria. Also, they have a broader host range than most plasmids. Conjugative transposons can transfer not only among species within the Gram-positive group or within the Gram-negative group but between Gram-positive and Gram-negative bacteria. Conjugative transposons were overlooked for a long time because they are located in the chromosome and thus cannot be isolated as easily as plasmids. Now that scientists are looking for them, they are finding conjugative transposons in many bacteria, including such familiar genera as *Salmonella, Vibrio, Rhizobium,* and *Agrobacterium.* Indeed, a recent study of resistance gene transfers among *Bacteroides* species in the human colon revealed that conjugal transfers of resistance genes occur much more frequently than expected (Box 16-3).

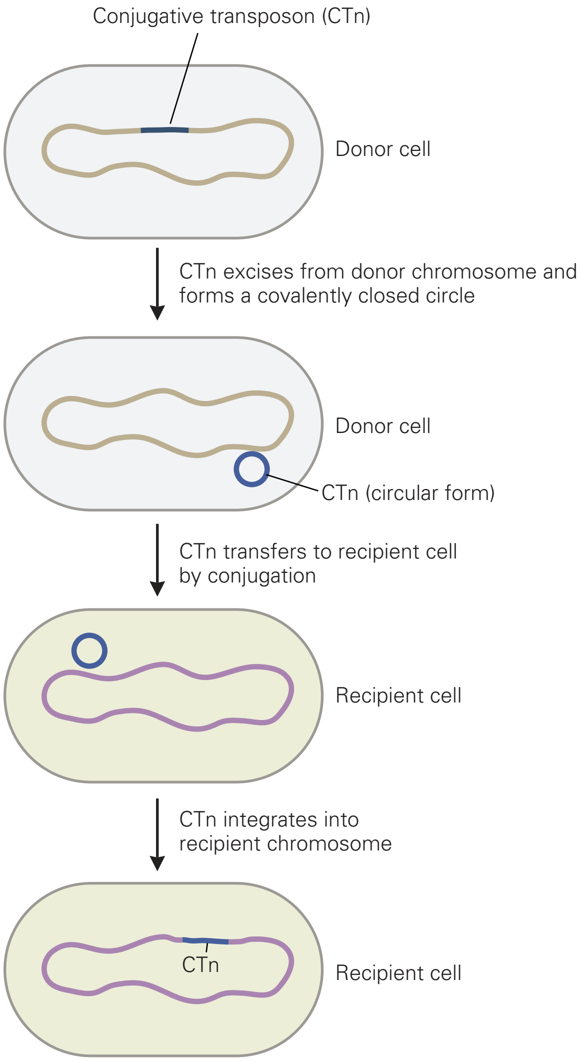
Conjugative transposon (CTn)
Donor cell
CTn excises from donor chromosome and forms a covalently closed circle
Donor cell
CTn (circular form)
CTn transfers to recipient cell by conjugation
Recipient cell
CTn integrates into recipient chromosome
CTn
Recipient cell

Figure 16-17. Transfer of a conjugative transposon. The transposon, which is integrated into the genome of the donor cell, excises itself to form a circular intermediate. The intermediate form transfers by conjugation into the recipient where it integrates into the recipient's genome.

Propagating and Maintaining Antibiotic Resistance through Selective Pressure and Changes in Fitness

Resistance can be genetically and biochemically complex and depends on the frequency at which the pathogen undergoes mutation and intra- or interspecies genetic exchange through HGT. Another confounding factor is the intensity of the selective pressure imposed on the pathogen by use of the drug. An inevitable side effect of the use of antibiotics is the emergence and dissemination of resistant bacteria. Thus, the question arises of whether removing the selective pressure (i.e., antibiotic use) will eliminate antibiotic resistance.

Vancomycin and third-generation cephalosporin use have been strongly correlated with a sharp rise in vancomycin-resistant enterococci (VRE) infections. A patient with VRE could be at increased risk of serious postsurgical infection that is difficult to treat. Of particular concern nowadays is the potential for spread of the vancomycin resistance to methicillin-resistant *S. aureus* (MRSA), which in a hospital setting is very problematic. Most health care officials believe that restricting antibiotic use will help limit the spread and development of more VREs. As such, it is now becoming common practice, particularly for hospitals, to campaign against overprescription of antibiotics by physicians, to implement more stringent hygiene practices such as frequent handwashing, and to strictly isolate patients that have VREs. The goal of this strategy is to reduce the transfer of the resistance genes by removing the selective pressure through reduction of medical usage of broad-spectrum antibiotics and through restriction of transmission. The problem with this approach is that it has been quite ineffective in halting the spread of VREs. Contrary to popular belief, the resistance genes never really go away once the selective pressure is removed. Indeed, the failure of the industry to demonstrate the effectiveness of including antimicrobials in soaps and body washes to prevent the spread of disease-causing microbes led the FDA to issue a final rule in 2016 that bans the use of one or more of 19 currently used active ingredients, including triclosan and triclocarban, from antibacterial soaps and body washes (hand sanitizers and antibacterial wipes are not affected by this rule).

There is no longer any doubt that subtherapeutic doses of antibiotics as growth stimulants for animals can select for resistant bacterial strains. Avoparcin, a vancomycin derivative, has never been approved for use in the United States, but it was used as a feed additive in Western Europe from 1986 until 1995. It was banned in 1995 due to the discovery that it was contributing to the selection of VanA-type VRE in animal husbandry. It was hoped that banning its use would reduce the potential for human exposure to this new VRE, but that has not been the case. There are noticeable differences in the epidemiology of VRE between Europe and the United States. European VREs are usually still susceptible to other antibiotics, most likely due to the overuse of avoparcin and the

Box 16-3.

How Common is Horizontal Gene Transfer of Resistance in Nature?

Conjugative plasmids or conjugative transposons freshly isolated from bacteria in nature generally transfer in the laboratory at very low frequencies. For instance, in a mating between a donor and a recipient, typically only one recipient in a million will acquire the plasmid or conjugative transposon. If this is the frequency of transfer under optimal laboratory conditions, one would think that transfer in nature, where conditions are presumed to be less ideal, is a very rare event, right?

To answer this question, one study focused on *Bacteroides* species in the human colon. *Bacteroides* is one of the numerically major bacterial genera in the human colon, accounting for about 25% of all colon isolates. *Bacteroides* species are known to have plasmids and conjugative transposons, both of which are capable of transferring antibiotic resistance genes. If the colon is perforated during surgery or other abdominal trauma, *Bacteroides* can cause life-threatening infections of tissue and blood. Alarmingly, this group of bacteria has become resistant to most antibiotics.

The scientists doing this study had access to a collection of *Bacteroides* strains that had been isolated before 1970 and to another collection of strains that had been isolated in the late 1990s. They found that before 1970, about 25% of the strains were resistant to tetracycline and none were resistant to erythromycin. By the late 1990s, over 80% of the *Bacteroides* strains were resistant to tetracycline and nearly one-third was resistant to erythromycin. By using DNA hybridization and DNA sequence analysis, the scientists were able to determine that the large increase in tetracycline resistance was due to a single resistance gene, *tetQ*, which had been spread to many different *Bacteroides* species. The increase in erythromycin resistance was due primarily to the spread of two genes, *ermF* and *ermG.* The DNA sequences of the *tetQ* and *ermF/ermG* genes found in different species were over 95% identical, a finding that suggested the genes had been transferred by HGT and did not arise by independent mutational events. Finally, the investigators were able to show that *tetQ* and probably the *erm* genes as well were carried on conjugative transposons. The results of this study suggested that HGT by conjugation occurs very readily in the human colon.

Source:

Shoemaker NB, Vlamakis H, Hayes K, Salyers AA. 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. *Appl Environ Microbiol* **67:**561–568.

subsequent emergence and spread of resistant strains that resulted specifically from its overuse. In contrast, VRE in the United States are resistant to many antibiotics, not just one. This is presumably due to hospital-acquired resistances from the overuse of various antibiotics in a hospital setting.

But why does resistance remain in bacterial populations, even when antibiotic use diminishes? This phenomenon results partly from the tremendous bacterial genetic plasticity, which is responsible for the development and spread of resistance in the first place. You may have wondered what happens to bacterial growth and physiology when bacteria pick up resistance mutations or genes. In general, resistance phenotypes initially make bacteria less "fit" compared to their nonresistant, sensitive parent strains. This makes sense, because many resistance mechanisms change the structures of macromolecular targets of the antibiotics. These changes often reduce the functions of these important cellular machines, and the resistant strain will grow and compete less well than the parent strain when the antibiotic is absent. But bacteria have two ways to cope with this fitness problem. As discussed earlier, many resistance mechanisms are tightly regulated so that the resistance genes are only turned on strongly when they are needed, as in the presence of an antibiotic. But bacteria also have other mechanisms to cope with constitutively expressed resistance. One common mechanism is to evolve their way out of the problem. For example, resistance mutations in rRNA, ribosomal proteins, DNA gyrase, or RNA polymerase often result in reduced fitness that initially decreases bacterial growth. Therefore, there is a window of time when the resistant bacteria may be outcompeted by the sensitive bacteria in the absence of antibiotic. However, optimal growth is a powerful genetic selection in itself, and the less fit resistant bacteria will begin to accumulate secondary mutations that restore growth to the resistant bacteria. These are not simple revertants, but rather compensatory mutations in the same gene (**intragenic**) or in other genes (**intergenic**) that restore growth. Thus, the window when sensitive bacteria can outcompete resistant bacteria can rapidly close, especially in large populations of bacteria such as those in sizable geographic locations. The outcome is the worst of both situations—entrenched resistant bacteria that are now as fit (or sometimes even more fit) than the starting sensitive bacteria.

Will We Return to the Pre-Antibiotic Era?

Returning to Status Quo or Moving Forward?

A scenario portrayed in some of the more lurid news stories about antibiotic-resistant bacteria is the return to a world much like that of the 1800s, when there were no antibiotics and people commonly died of diseases like pneumonia and wound infections. Surgery would once more become an intervention of last resort with a high mortality rate due to untreatable postsurgical infections. Is this likely to happen? Well, probably not. First, it is highly unlikely that all antibiotics will become ineffective against all bacteria. It is also unlikely that all bacteria will become resistant to all antibiotics, although there are now a few rare examples and a few more that are very close. Interestingly, there are some bacteria, such as *Streptococcus pyogenes*, a dreaded cause of wound infections and strep throat in children, that have so far remained susceptible to most antibiotics, including β-lactam antibiotics. Why this continued susceptibility is seen in some—although unfortunately not many—bacteria is not clear, but it gives room for hope that there will still be some treatable diseases.

A second consideration is that we have learned a lot about bacterial infections since the 1800s. Passive immunization is currently approved to treat infections caused by a number of toxin-producing bacteria (more on this in chapter 17). In this form of immunotherapy, serum or immunoglobulin fractions are isolated from humans or horses that have been inoculated with a specific antigen. These antibody fractions can be injected into a person and provide limited protection against infections in which the antigens play critical roles. It is feasible that passive immunization could be extended to treat infections caused by highly drug-resistant bacteria, such as *S. aureus*. For certain high-risk groups, such as the elderly and the very young, vaccines have offered significant protection against some bacterial pathogens, such as the capsule and conjugated-capsule vaccines directed against *S. pneumoniae*.

A third consideration is that advances continue to be made in surgical procedures that make them less invasive. Laser surgery, which does not create huge surgical wounds, is not nearly as conducive to the development of postsurgical infections as cut-and-stitch surgery. Good hygienic practices such as handwashing and sterilizing surfaces will continue to prevent many infections, if these practices are observed rigorously. As long as we do not lose disinfectants, a great deal can be done to prevent infections from occurring in the first place. And bacteria, inventive little devils that they are, are not likely to become resistant to autoclaving. There may be other preventive measures that allow people to protect themselves from developing the predisposing conditions that increase the risk of infection. People with uncontrolled diabetes are one such high-risk group for bacterial infections. Improvements in control of diabetes do reduce this risk. Similarly, new methods for bolstering the flagging immune systems of the elderly may make them less vulnerable to disease.

The much-maligned pharmaceutical industry should not be overlooked. Although current market forces discourage antibiotic discovery and development, the pharmaceutical industry has had a brilliant past record of finding and marketing new antibiotics. Finding ways to speed up the approval of new antibiotics may be needed in some cases. However, this could mean that the public is going to have to accept a higher level of risk of side effects if the clinical trials that cost so much time and money are abbreviated. Faced with the alternative of dying, however, people may find that side effects are acceptable after all. AIDS and cancer patients have already made this psychological transition from insisting on completely risk-free treatments to a willingness to take chances on new therapies.

Finally, technological and scientific advances are dramatically and quickly expanding our understanding of the mechanisms by which antibiotics work and how resistance develops and evolves. Although first passes at applying combinatorial chemistry, structure-based design, genomics, and robotic screening were not as successful in antibiotic discovery as initially hoped, these approaches are very much in their infancy and still have huge potential to discover new antibiotics and modify existing ones. Many older natural products with antibiotic activity that were previously passed over might now be revisited using new microbiological and chemical approaches. Antibiotic combinations also hold a new promise to treat emerging and re-emerging diseases.

This brings us back to a theme that was introduced in chapter 4 on host defenses: the human mind is one of the most important defenses against disease. If scientists and the public put their minds (and resources) to conquering the worsening resistance problem instead of ignoring it as they have until recently, surprising and wonderful things could happen. For example, the new teixobactin class of natural product antibiotics was recently discovered by devising methods to culture colonies of soil bacteria that do not grow on common laboratory media. This method involved a device, called an iCHIP, that allows bacterial growth in the soil environment. Screening of separate colonies for antibiotic activity led to the discovery of teixobactin, which binds to lipid II and inhibits peptidoglycan biosynthesis in Gram-negative bacteria. So far, resistance has not been observed to teixobactin, although there is considerable speculation that some mechanism of teixobactin resistance may eventually emerge.

One of the biggest casualties of bacterial diseases that become incurable may be confidence in the medical establishment. The public is disgruntled because scientists have not come up with better cures for cancer or for HIV infection, but at least we were never in a position in the past to cure these diseases. How will the public react if a point comes when parents have to watch children die of infections that were once curable? No one knows what the psychological fallout of lost cures will be. Moreover, as parts of the developing world, which were largely left out of the antibiotic and vaccine revolutions, become more prosperous, how will people in those countries feel about those in developed countries who squandered the miracle drugs people in developing countries are now in a position to afford?

It does not take a rocket scientist to figure out that part of the solution is not to lose the antibiotics we already have, but instead to conserve this precious resource by curbing the reckless abuse and overuse of antibiotics (especially those precious drugs of last resort) by the medical profession and agricultural industry. We do not need images of skeletons, such as those that graced the cover of popular science magazines, to evoke images of a coming plague. Instead, we need images of responsible behavior by patients, physicians, and industries dedicated to the preservation of antibiotics for the next generations. We also need a periodic supply of new antibiotics and vaccines to set back the clock in the relentless race against the evolution and spread of bacterial resistance, which has been all but inevitable up to this point in time.

The Hunt for Alternative Approaches to Antibiotics

What will we do when we no longer have any drugs of last resort available? In anticipation of such a dire situation, a number of research groups are seeking alternative strategies to overcome the problem of relying on antibiotics. While many of these approaches are still in their infancy, we will mention here a few of these interesting new research directions.

Probiotics. This approach takes advantage of the "good" resident microbiota to outcompete any pathogens that might be introduced into a microbiome by preventing attachment and colonization and by taking nutrients away from the pathogen. Probiotics are generally defined as live microbes that confer a health benefit to the host when administered in appropriate amounts and proportions. This approach has begun to be applied to diseases that are classified as polymicrobial- or community-based or as microbial-shift diseases (see chapter 5). Bacterial vaginosis and inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, are commonly thought to be conditions that result from an imbalance of the normal microbiota due to a loss of "good" bacteria and an overpopulation of other "bad" bacteria. The premise of oral intake of probiotics in these cases is that this will reintroduce the missing "good" bacteria, such as *Bifidobacteria* for the gut system or *Lactobacilli* for the vaginal system. The presumption is that these bacteria will help restore the normal microbial composition of the microbiome without the need to use antibiotics to remove the detrimental bacteria.

Bacteriophage or Bacteriolysin Therapy. This approach takes advantage of the killing power of specific bacteriophages that attack and lyse only certain types of bacteria with specific phage receptors, while leaving the other "normal" resident microbiota alone. Bacteriophage therapy was first developed for streptococcal and staphylococcal infections, but it has now been applied for other pathogens, including *Bacillus anthracis*. The premise of this strategy is that the lytic bacteriophages will kill the entire bacterial population that they infect within a very short period of time. One concern with this approach is the safety of using an agent that can propagate so rapidly. Bacteria can evolve to resist phages, but, vice versa, phages can also evolve. The fear is that the phages could evolve to kill other bacteria besides the ones originally intended. Nevertheless, a number of biotechnology companies have formed with the goal of developing this therapy.

A strategy to avoid issues without relying on whole bacteriophages is instead to develop the key enzymes that phages use to lyse bacteria, the phage lysin. Each phage strain produces a bacterial strain-specific lysin that it uses to disrupt the bacterial cell wall through its peptidoglycan hydrolase activity and escape by causing cell lysis. Lysins work very rapidly to kill the bacteria (Figure 16-18) and, like the phages that encode them, they often have relatively narrow host-range specificity.

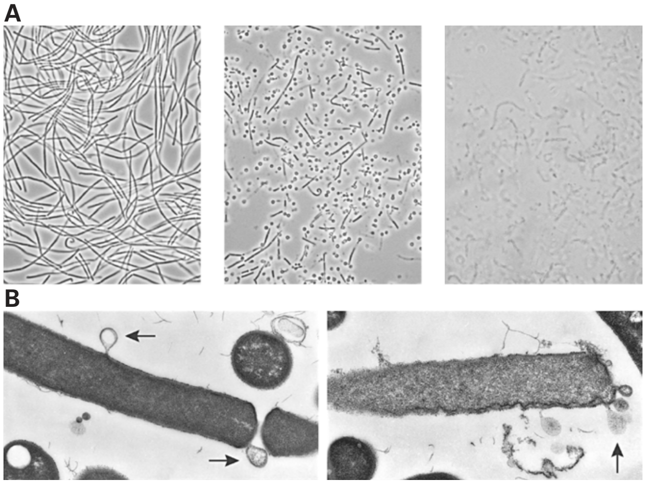

Figure 16-18. Bacteriophage therapy. Shown is a time course of treatment of *Bacillus cereus* bacteria with bacteriophage lysin. **(A)** Phase-contrast images of *B. cereus* before (left panel), after 1 minute (middle panel), and after 15 minutes (right panel) treatment with the lysin. **(B)** Electron micrographs of *B. cereus* show membrane blebbing (arrows) has already begun after 1 minute of treatment with lysin (left panel) and extensive bacterial cell damage has occurred after 10 minutes of lysin treatment (right panel). Reproduced from Thiel K. 2004. *Nat Biotechnol* **22:**31–36, with permission.

Anti-Adhesion Therapy. The basis of this approach is to interfere with pathogen colonization and/or invasion of the host by blocking its ability to bind to cell surfaces. As we will see in chapter 17, active or passive vaccination against bacterial adhesins still serves as a viable and, in many cases, a highly effective means of preventing bacterial infections by producing antibodies that target surface adhesins of the bacteria and block bacterial attachment and uptake into host cells. Another method to block bacterial adherence is to target the expression of receptors on the pathogen by inhibiting their biogenesis, such as using **pilicides** to prevent pilus assembly. Diguanylate cyclase, which makes the bacterial second messenger cyclic-di-GMP involved in regulating biosynthesis of adhesins and exopolysaccharide matrix components for biofilms, is an attractive target for drug development. Alternatively, expression of receptors on the host could be targeted by inhibiting their biogenesis, including methods such as depleting certain glycosphingolipids through treatment with drugs that block biosynthetic enzymes in the pathway.

Because many bacterial adhesins interact with sugar groups of surface molecules on the host cell, sugar-based inhibitors can be used to interfere with binding of the adhesin to its cell surface receptors, either by binding tightly to the bacterial adhesin or by binding tightly to the host cell receptor. An example of this strategy is the unique agglutinating lectins LecA and LecB, from *P. aeruginosa*, which are small proteins of 121 and 115 amino acids, respectively. Both lectins are important bacterial adhesins that bind to bacterial polysaccharides in biofilms and to human glycoproteins; LecA binds D-galactose and N-acetyl-D-galactosamine, while LecB binds L-fucose, D-mannose, D-arabinose, D-fructose, and a few other sugars. Many of these monosaccharides can bind to *P. aeruginosa* with higher affinity than polysaccharides or glycoproteins, and so addition of solutions containing these sugars can block bacterial adherence and biofilm formation, and thereby reduce infection.

One notable drawback of this approach is the general redundancy that is observed for many pathogens. Indeed, bacterial pathogens often have multiple types of adhesins that are differentially expressed during the course of infection, which are also dictated by the multiple routes of infection and the sites within a body where they disseminate. Similarly, the host cell receptors that the bacteria encounter also change depending on where in the body they are located. This variability hampers development of an adhesin-based inhibitor that works in all cases and during the entire course of the infection.

Blocking Quorum Sensing and Global Virulence Regulation. Since quorum sensing regulates many virulence traits, such as toxin production and biofilm formation, one alternative antibacterial strategy is to interfere with quorum sensing. Targets that are being actively pursued include inhibiting the production of the autoinducer signaling molecule, degrading the autoinducer, disrupting the sensor kinase-response regulator system, and using interfering autoinducers from competing bacterial strains. Along these lines, several small molecule inhibitors that block quorum sensing in a number of pathogens have been discovered by high-throughput screening efforts.

Global virulence is another area that is targeted for drug screening. The premise here is that the drugs could interfere with production of the virulence factors (such as toxins) or biofilm formation even if they do not inhibit growth or kill the bacteria. Virstatin (Figure 15-26) is an example of one such drug candidate against *Vibrio cholerae* toxin production that was identified through high-throughput virulence screening. Two-component regulatory systems and regulatory RNAs (see chapter 14) are also considered attractive targets for drug screening efforts. Several small molecule candidates have been identified so far and are currently undergoing further testing.

The challenge encountered with these strategies is the rapidity with which the bacteria can mutate the target proteins or their expression levels, particularly since they are often not essential for bacterial growth or survival in the environment. Using these targets also means that the resulting drug candidates have very narrow activity spectrums, since they are usually present in a limited range of bacterial species. A final potential drawback of these approaches is that because the drugs do not kill the bacteria, their effectiveness and use in immunocompromised patients may be limited.

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Questions

1. Explain how mutant porins could help make β-lactamases more effective. Could mutant porins team up with other types of resistance mechanisms to increase the effectiveness of the resistance mechanism?
2. Efflux pumps seem very energy inefficient. Why don't bacteria simply fail to take up antibiotics into the cytoplasm?
3. Efflux pumps only reduce the level of an intracellular antibiotic; they do not eliminate the antibiotic completely. Why does this make the bacteria resistant to the antibiotic?
4. Some target modifications require only one or a few mutations (e.g., resistance to fluoroquinolones). Yet others, such as protection of the ribosome by methylation of rRNA, require new enzymes. Explain the difference.
5. Why does mutation to resistance occur within a short time in some cases (e.g., erythromycin or penicillin) and only over a period of decades in other cases (e.g., vancomycin)?
6. In the case of Gram-negative bacteria, efflux pumps are generally coupled to outer membrane proteins. Why is this necessary?
7. Can you think of a type of resistance mechanism that is possible in theory, although it has not yet been found?
8. Why can transfer of DNA by conjugation cross genus lines, whereas transfer of DNA by natural transformation or phage transduction is usually limited to a few closely related organisms?
9. Some integrons have been found that contain disinfectant-resistance genes or mercury-resistance genes as well as antibiotic-resistance genes. What is the potential significance of this association?
10. Give a possible set of bacterial factors (or lack thereof) and what properties they have that would explain an increase of the IC_{50} value (50% inhibitory concentration) for tetracycline by a thousandfold in an organism.
11. You are characterizing a mutant of *Staphylococcus aureus*. The mutation affects the cell wall such that the cells make only a thin layer of peptidoglycan (making up about 20% of the cell wall). What would you expect the result to be for treatment with each of the following antibiotics?
    a. penicillin
    b. tetracycline
    c. erythromycin
    d. vancomycin
12. Indicate which of the following statements are TRUE or FALSE:

______ Antibiotic resistance is a consequence of the mutagenic action of the antibiotic on the bacterium.

______ Antibiotic resistance is clinically important because it results in increased toxicity.

______ Antibiotic resistance is clinically important because it results in increased morbidity and mortality.

______ Antibiotic resistance is clinically important because it results in increased costs.

______ Misuse of antibiotics has resulted in greater numbers of antibiotic-resistant organisms.

______ The patient's normal microbiota can serve as a reservoir for antibiotic-resistance genes.

13. Which of the following is the easiest way for bacteria to develop antibiotic resistance?
    a. Generate a mutation to alter the target protein.
    b. Add a functional group to the antibiotic.
    c. Acquire a conjugative plasmid harboring an antibiotic-resistance gene.
    d. Mutate the gene that regulates the antibiotic-resistance genes.
    e. Mutate the efflux pump so that it pumps out the antibiotics faster than they can accumulate in the bacterial cell.
14. Bacteria can become resistant to fluoroquinolones by producing a mutation in what protein?
15. Fill in the blank spaces with the most appropriate words:

    Some members of enterococci are resistant to _________, a last-resort antibiotic that binds to the __________ dipeptide of N-acetyl muramic acid within the peptidoglycan.

    The enterococci develop resistance by replacing this dipeptide with ________, which is not bound by the antibiotic. A novel way to overcome this resistance is to ________.

    The most common way antibiotic-resistance genes are spread is by ___________.

    The main source of antibiotic-resistance genes is believed to occur through ___________ between bacteria found in the ___________.
16. How might a mutant porin in a Gram-negative bacterium allow the bacterium to become less susceptible to antibiotics? How could this mutant porin help to make β-lactamases and efflux pumps more effective?

17. Enzymes such as acetyltransferases, phosphotransferases, or adenyltransferases would inactivate which of the following antibiotics?
    a. methicillin
    b. streptogramin
    c. chloramphenicol
    d. ciprofloxacin
    e. tetracycline
    f. vancomycin
    g. erythromycin
    h. penicillin

18. Production of a new penicillin-binding protein would reduce the affinity for which of the following antibiotics?
    a. methicillin
    b. streptogramin
    c. chloramphenicol
    d. ciprofloxacin
    e. tetracycline
    f. vancomycin
    g. erythromycin
    h. penicillin

19. What does the XDR stand for in XDR tuberculosis?

20. Which is resistant to more drugs: MRSA or VRSA?

Solving Problems in Bacterial Pathogenesis

1. Recent headlines have highlighted the spread of MRSA infection in the United States. According to the CDC, MRSA is responsible for over 90,000 serious infections and over 18,000 hospital-stay-related deaths per year in the United States. MRSA is also responsible for many serious skin and soft tissue infections as well as pneumonia. One major problem with MRSA is that occasionally the skin infection can spread to other organs of the body with more severe, life-threatening symptoms, including necrotizing fasciitis (tissue destruction) and necrotizing pneumonia (hence the name flesh-eating bacteria), followed by sepsis and toxic shock, then death in up to 50% of cases. A striking finding about these infections is that they even occur in young immunocompetent patients who were previously healthy. MRSA is resistant to several commonly prescribed antibiotics that are usually effective against Gram-positive bacteria (methicillin, penicillin, and cephalosporins), and an infection with a MRSA strain can be deadly if left untreated. MRSA is subcategorized as either community-acquired (CA-)MRSA or hospital-acquired (HA-) MRSA, depending on how the infection is usually acquired. Most CA-MRSA strains are still sensitive to many antibiotics, such as trimethoprim, tetracycline, and clindamycin, but HA-MRSA strains are often resistant to these drugs while still sensitive to vancomycin and linezolid.
    a. Provide a common mechanism that accounts for the observed resistance of MRSA to methicillin, penicillin, and cephalosporin. Provide two different strategies that could be used to overcome this particular resistance.
    b. For each of the antibiotics trimethoprim, tetracycline, and clindamycin, provide a possible mechanism to account for the observed resistance of HA-MRSA to that antibiotic. Provide a strategy that could be used to treat patients infected with HA-MRSA resistant to these antibiotics.
    c. Why would HA-MRSA strains that are resistant to methicillin, penicillin, cephalosporins, trimethoprim, tetracycline, and clindamycin still show sensitivity to vancomycin and linezolid?
    d. Although CA-MRSA is resistant to clindamycin, treatment with clindamycin in combination with rifampin results in an increase in the 50% lethal dose (LD_{50}) value from 10 without antibiotic treatment to 10^4 with rifampin and to 10^8 for the combined treatment with clindamycin and rifampin. In addition, treatment with clindamycin enhanced opsonization of CA-MRSA by macrophages. What possible mechanism(s) could account for these observations (i.e., change in LD_{50} value and enhanced opsonization)? Provide your rationale. Provide an experiment that could be performed to confirm your hypothesis.

Special Global Perspective Problems: Integrating Concepts in Pathogenesis

Problem 1

A researcher ordered a colony of rabbits for his studies. Shortly after arrival at the animal care facility, however, the university veterinarian discovered that most of the animals in the colony had contracted a bacterial infection. Clinical signs were generally limited to chronic infection of the lungs in the healthy adult animals, but young animals succumbed to systemic infection, including brain lesions and death. The veterinarian and researcher enlisted your help to study the cause of this disease. Subsequently, you isolated a new virulent Gram-positive bacterium related to *Listeria monocytogenes*, which you named *Listeria leporine*.

Using a Tn-Seq-based approach using existing reagents already developed for *L. monocytogenes*, you identified eight genes encoding putative virulence factors, which you named *llp1* through *llp8*. You then created mutant strains with in-frame deletions in each of these eight genes. When administered to rabbits through a breath nebulizer, the wild-type bacterium has an ID$_{50}$ value of 10 for lung colonization in young and adult animals, whereas it has an LD$_{50}$ value of 10 for systemic infection with brain lesions in young animals but an LD$_{50}$ value of 10^6 for adult animals. Mutant Δ*llp*4, Δ*llp*7, and Δ*llp*8 have LD$_{50}$ values in young animals similar to those in the wild type but have ID$_{50}$ values for lung colonization of 10^7. On the other hand, Δ*llp*1, Δ*llp*2, Δ*llp*3, Δ*llp*5, and Δ*llp*6 have LD$_{50}$ values in young animals of $>10^9$ but have ID$_{50}$ values for lung colonization similar to those of the wild type. To help in your studies, you have generated antibodies against each of the proteins in order to visualize your proteins in Western blots. You have also developed an *in vitro* cell culture infection model using rabbit lung cells and have determined that *L. leporine* invades the rabbit cells and enters a phagosome, but then escapes from the phagolysosome to replicate in the cytosol. Similar to *L. monocytogenes, L. leporine* also appears to move about in cells using actin filaments that gather at one end of the bacterium (forming what looks like a comet tail) as a means for propulsion.

1. You determine that cationic peptides, such as those released into the phagosome upon fusion with a lysosome, stimulate expression of the *llp1, llp*2, and *llp3* genes that are deleted in Δ*llp*1, Δ*llp*2, and Δ*llp*3, respectively, but inhibit the expression of the *llp*4 gene that is deleted in Δ*llp*4. Describe how you could determine if the expression of the *llp*1–*llp*4 virulence factors in the presence of cationic peptides is regulated at the transcriptional level. Be sure to include your expected results.

2. Δ*llp*5 and Δ*llp*6 are no longer stimulated by cationic peptides to express the *llp*1, *llp*2, and *llp*3 virulence factors, whereas *llp*4 is expressed. Cellular fractionation revealed that Llp5 is cytoplasmic and Llp6 is membrane-bound. Sequence analysis shows that Llp6 has four hydrophobic helices and an ATP-binding motif in its C terminus.
   a. Predict (with rationale) a function for the proteins deleted in Δ*llp*5 and Δ*llp*6.
   b. Provide (with rationale) an experiment to confirm your prediction for each protein.

3. Despite numerous attempts, you discover that you cannot visualize the Llp4 protein in Western blots of bacterial cells, yet you were able to observe transcriptional regulation of the *llp*4 gene by cationic peptides. After further analysis of the *llp*4 sequence you discover that part of the sequence is complementary to the 5′ promoter regions of several polysaccharide biosynthetic genes and a couple of adhesin-like genes. Provide (with rationale) a possible explanation for these observations.

4. You find that Δ*llp*1 and Δ*llp*2 can no longer escape from the phagolysosome, whereas Δ*llp*3 can still escape into the cytosol. However, Δ*llp*1, Δ*llp*2, and Δ*llp*3 are all no longer able to make the actin tails. Sequence comparison with protein databases revealed that the gene *llp*1 encodes a 100-kDa protein with homology to pore-forming cytolysins, while *llp*2 encodes a 50-kDa protein with homology to phospholipases and *llp*3 encodes a 20-kDa protein with no homology to any known protein. All three proteins have approximately 20 hydrophobic amino acids at the N-terminus. You perform a series of cellular fractionations to determine the localization of the proteins. In the first fractionation, you examine wild-type bacterial cells cultured in medium without or with cationic peptides. In the second fractionation, you examine lung cells cultured with bacteria and then lysed with a detergent that does not lyse bacterial cells, followed by centrifugation to pellet bacteria (denoted as pellet) and collection of the supernatant fraction (denoted as lysate). You then run SDS-PAGE to separate the proteins, followed by Western blot analysis.
   a. Provide a detailed interpretation (with rationale) of the results shown in Figure 1.

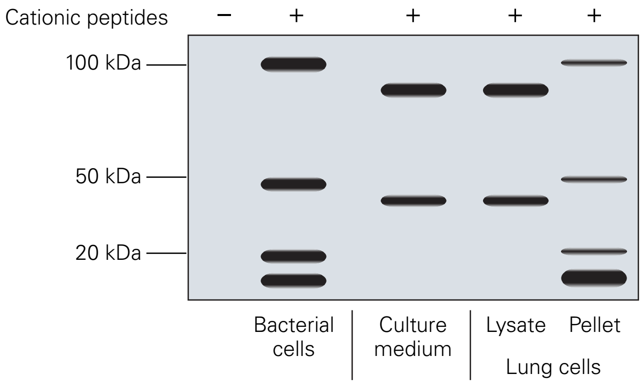

Figure 1. Western blot results.

   b. Predict (with rationale) a possible function for each of the Llp1, Llp2, and Llp3 proteins.
   c. Provide an experiment that could be performed for each protein to confirm your predictions.

5. Curious about the possible source of the contamination in the rabbit housing facility, you find that water samples from several of the water bowls in the animal cages near an air-handling vent inlet are indeed contaminated with the bacteria, and you then find that there is a biofilm of the bacteria coating the moist inside of the vent. When you examine the bacteria from fresh isolates obtained from the vents, you notice that like the bacteria

from fresh lung isolates but unlike the bacteria obtained from blood or brain lesions, the colonies are mucoid with a thick polysaccharide capsule. Using the rabbit lung inhalation model, you infect the animals with wild-type and mutant bacteria for one week. You find that wild-type bacteria and Δ*llp*1, Δ*llp*2, Δ*llp*3, Δ*llp*5, and Δ*llp*6 mutants can all be isolated easily from the lungs of the animals with mucoid phenotype, but Δ*llp*4, Δ*llp*7, and Δ*llp*8 cannot be isolated from lungs after one week but instead can be isolated from brain lesions of young animals that die and are not mucoid.

a. Although the wild-type bacteria lost their mucoid phenotype after plating on agar overnight, you find that culturing the wild-type bacteria in broth for a few days restores the mucoid character. You also find that if the medium from old cultures is added to the bacteria lacking polysaccharide on their surfaces, within a short time those bacteria begin to express polysaccharide on their surfaces. Provide a mechanism for both the *in vitro* and *in vivo* (in lungs and in brain) observations regarding capsule.
b. You found that the genes deleted in Δ*llp*7 and Δ*llp*8 are located within the same operon comprised of four genes, which you have tentatively named *llp*7, *llp*8, *llp*9, and *llp*10. Sequence analysis revealed that *llp*7 encodes a 100-amino-acid peptide with an N-terminal hydrophobic region; *llp*8 encodes a 400-amino-acid protein with five hydrophobic regions and a C-terminal nucleotide-binding motif; *llp*9 encodes a 300-amino-acid protein with three hydrophobic regions and a C-terminal nucleotide-binding motif; and *llp*10 encodes a 200-amino-acid-protein with a DNA-binding motif. You make additional Δ*llp*9 and Δ*llp*10 deletion mutants. You observed that old medium from wild type, Δ*llp*4, Δ*llp*8, or Δ*llp*10 added to nonmucoid cultures of Δ*llp*7 or Δ*llp*9 restores the mucoid phenotype, but when old medium from Δ*llp*7 or Δ*llp*9 is added to nonmucoid cultures of wild type, Δ*llp*4, Δ*llp*8, or Δ*llp*10 the mucoid character is not restored. Provide a possible explanation for these observations. Draw a clearly labeled schematic diagram depicting the location of each gene product (for *llp*4, *llp*7, *llp*8, *llp*9, and *llp*10) and its possible role in regulation of the mucoid phenotype.
c. You find that although the mutant strains Δ*llp*4, Δ*llp*7, and Δ*llp*8 have LD_{50} values in young animals similar to those of wild-type bacteria, they are now sensitive to treatment with erythromycin, azithromycin, tetracycline, kanamycin, and chloramphenicol, and these antibiotics can be used to treat infection, whereas wild-type bacteria and the other mutant strains are still resistant to the antibiotics and cause chronic lung infections. Provide (with rationale) a possible explanation for this observation.

Problem 2

You have isolated a new bacterium from a deadly hospital outbreak in 2015 that, based on 16S rRNA comparison, is a strain of the Gram-negative opportunistic pathogen *Acinetobacter baumannii* (Ab). Ab strains are notorious MDR/XDR nosocomial (hospital-acquired) pathogens able to colonize patients in intensive care units, usually causing pneumonia, septicemia, and/or meningitis with high morbidity and mortality rates. Interestingly, your Ab isolate was obtained from a patient with a urinary tract infection (UTI) that, despite aggressive antibiotic treatment, progressed to hemolytic-uremic syndrome (HUS involves bloody urine and kidney failure), followed by septicemia and death. You have determined that your clinical isolate Ab15 is resistant to all aminoglycosides (such as kanamycin and gentamicin), all β-lactams (including clavulanic acid but not carbapenems), chloramphenicol, tetracyclines, ciprofloxacin, trimethoprim, and the dye ethidium bromide. Using Tn-Seq technology in a mouse model of UTI, you have identified four potential operons that may encode for virulence factors. There are several whole genome sequences available for other MDR and XDR Ab strains.

1. You find that three of the operons identified appear to be part of a regulon. What specific evidence might have led you to conclude this? Describe an experiment that could be performed to identify all operons that are upregulated during UTI. *Briefly* describe how you would conduct this experiment.
2. One operon consists of two genes in tandem, which you have named *antBA*. Sequence comparison with protein databases reveals that *antA* encodes a protein of 85 kDa and has significant sequence similarity in its N-terminus with the catalytic domain of diphtheria toxin, while *antB* encodes a protein of 20 kDa and has significant sequence similarity with the B subunit of cholera toxin. Unlike wild-type bacteria, deletion mutants of *antBA* no longer kill cultured mouse bladder cells or caused HUS in mice. You make antibodies against the culture filtrate from the wild-type Ab15 cells and use the antisera in Western blots of the cell lysates from the wild-type bacterial cells, the bacterial culture medium, and the bladder cells exposed to bacterial cell-free medium.
   a. Provide a detailed explanation (with rationale) and interpretation for the results shown in the Western blot in Figure 2.
   b. Draw a diagram depicting a possible model of action that accounts for all of the experimental observations. Be sure to justify your model (i.e., it must be consistent with your interpretation of the data).

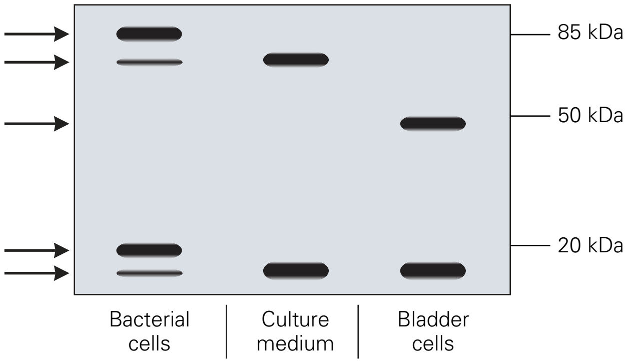

Figure 2. Western blot results.

3. Based on the above information, you propose that AntBA is responsible for the HUS disease symptoms caused by Ab15 strains.
    a. What criteria must be satisfied to definitively link infection with Ab15 to HUS? Be sure to state these criteria in terms of this specific case.
    b. Based on the available information, which of these criteria have been satisfied and which have not? Be sure to include your rationale.
4. You believe that AntA and AntB would make good candidates for a component vaccine to prevent HUS by Ab15 strains.
    a. What two pieces of evidence led you to propose these proteins as potential vaccine candidates?
    b. Your colleague believes you have made a mistake and that only one of the proteins would make a good vaccine candidate. Which protein did your colleague suggest is the better candidate? What was your colleague's most likely rationale for making this conclusion?
    c. Describe in detail how the antigens from the chosen vaccine component would be presented to the immune system to provide long-lasting protective immunity.
5. In further experiments to determine what regulates the production of AntBA, you find that both AntA and AntB are secreted into the medium only when the bacteria are grown under *in vivo* conditions, such as high temperature (37°C) and high CO_2 levels in the mouse UTI model or in overnight bacterial cultures, but not on agar plates or in fresh dilute culture medium. A second operon contains two genes (*antCD*) and when you knock the genes out with a transposon insertion, you find that AntA and AntB are no longer made *in vivo*, and the mutants are no longer resistant to kanamycin or gentamicin.
    a. Describe in detail how you would determine quantitatively if *antBA* gene expression is regulated transcriptionally under physiological conditions by AntC and/or AntD. Be sure to include expected outcomes.
    b. When you add overnight bacterial culture filtrates from wild-type Ab15 or from the *antC* deletion mutant to the *antD* deletion mutant cultures, you find that antibiotic resistance and AntBA production is restored, but adding the culture filtrates from the *antD* deletion mutant to the *antC* deletion mutant cultures does not restore antibiotic resistance or AntBA production. What possible mechanism could account for these observations? Give details and be specific! Be sure to predict the function of AntC and AntD.
6. The third operon consists of five genes (*antEFGHI*). The *antEF* and *antGHI* genes are adjacent but separated by an overlapping promoter region in which the sets of genes are transcribed in opposite directions. You discover that transposon insertion into any of the *antEFGHI* genes results in loss of antibiotic resistance to chloramphenicol, tetracyclines, ciprofloxacin, and ethidium bromide. Analysis of the sequence of *antE* reveals that it encodes a protein predicted to have four membrane-spanning hydrophobic sequences in its N-terminal domain and some sequence homology to protein kinases in its C-terminal domain. Sequence analysis of the other genes reveals that none of them encode proteins with any strong sequence homology to known proteins in the database but predicts the following: AntF does have a helix-loop-helix secondary structure motif that is frequently found in DNA-binding proteins; AntE has an ATP-binding motif and 12 membrane-spanning hydrophobic sequences; AntG and AntI also have multiple stretches of hydrophobic residues; and AntH has no strongly hydrophobic regions. You make antibodies against each of these proteins. In order to determine the cellular localization of the proteins, you fractionate the bacteria into cytoplasmic, inner membrane, periplasmic, and outer membrane fractions. You find that AntF is located in the cytoplasm, AntE and AntI are in the inner membrane, AntH is in the periplasm, and AntG is in the outer membrane.
    a. Based on the experimental results, draw a schematic diagram of a model of the bacterium and the location of the AntEFGHI proteins that accounts for all of the observations and possible mode of action.
    b. Provide an interpretation of the results that accounts for all of the observations.
7. The fourth operon consists of nine genes (*antJKLMNOPQR*) that are unique to Ab15 and are not found in other known Ab strains. The *antJK* and *antLMNOPQR* genes are adjacent but separated by a promoter region

flanked by inverted repeats. You find that fresh isolates of wild-type bacteria from mouse infections have fimbriae on their surfaces and the promoter is the direction of the *antLMNOPQR* genes, while bacteria grown continuously on agar plates lack fimbriae on their surfaces and have the promoter in the direction of the *antJK* genes. Sequence analysis of the genes reveals that the *antJK* genes encode proteins predicted to be DNA recombinases, and the *antLMNOPQR* genes encode proteins involved in Sec-dependent biosynthesis of type 1 fimbriae, where AntL is the major pilus subunit, AntM is a cytoplasmic chaperone, AntN is the outer membrane usher that assembles the pilus, AntP and AntQ form the pilus tip, and AntR is the mannose-binding adhesin. Deletion of any of the *antLMNOPQR* genes results in loss of virulence in the mouse UTI model.

a. Interpret these results in the context of Ab15 pathogenesis (compared to other Ab strains).
b. Provide an explanation for the observed promoter switching. Why is this important in the context of Ab15 pathogenesis?
c. You propose that the fimbriae encoded by this operon are important for adherence to mannose-containing receptors on bladder cells. Provide an additional different experiment that would definitively confirm your prediction.

17

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CHAPTER 17

Vaccination

A CRITICAL COMPONENT OF THE MODERN MEDICAL ARMAMENTARIUM

Edward Jenner (1749–1823) introduced the first vaccine in 1797, a cowpox vaccine that protected millions of people from the dreaded human disease smallpox and led to the eradication of the disease 180 years later. You would think that the vaccine would have been greeted with jubilation and gratitude—and indeed, it was supported by a number of influential people at the time who promoted its use. However, there was also considerable ambivalence and public criticism, as exemplified by a famous cartoon print from 1802 by the English satirist James Gillray (1756–1815) that portrayed people who had been vaccinated as having small cow heads sprouting from various areas of their bodies. Fortunately, many more successful vaccines have since appeared and have had major beneficial impacts on public health worldwide. Yet, even today, there are still some pockets of ambivalence, such as parents who refuse to vaccinate their children because they believe, despite overwhelming evidence to the contrary, that certain vaccines cause neurological diseases, such as autism. As the old saying goes, the more things change, the more they stay the same.

Vaccines: A Major Health Care Bargain

The concept of **acquired immunity** has been around for a long time. In his account of the plague of Athens during the second year of the Peloponnesian War (ca. 430 B.C.E.), the ancient Athenian historian Thucydides described how individuals who recovered from the plague were pressed into caring for the sick and burying the dead because it was well known that they would not develop disease again; that is, they had acquired immunity to the plague. Two millennia later, **variolation** (subcutaneous inoculation or scarification of unimmunized individuals with a tiny amount of material from a smallpox pustule of a diseased patient) was commonly practiced in many parts of the world to prevent large smallpox outbreaks. While most of the time this process of using a tiny amount of fully virulent smallpox virus was successful in causing only a mild infection, there was still a significant (2–3%) risk of the inoculated individual coming down with severe disease and dying or transmitting the disease to others.

In 1797, Edward Jenner (1749–1823) demonstrated the concept of acquiring protective immunity against smallpox using material that he had taken from the pustules of milkmaids with cowpox, a less virulent relative of smallpox. He dubbed this process of immunization against smallpox by inoculation with cowpox as **vaccination** (*vacca* being Latin for cow). A century later Louis Pasteur (1822–1895) extended the concept of vaccination to include gaining protective immunity against other infectious agents, such as bacteria (fowl cholera vaccine in 1880 and anthrax vaccine in 1881) or other viruses (rabies vaccine in 1885), through inoculation with attenuated versions of those pathogens.

Today, for safety, all **vaccines** are comprised of nontoxic or greatly attenuated antigens, which are deliberately injected, ingested, or inhaled to induce a specific, protective immune response (a process referred to as **active immunization**) without having to go through the more serious, harmful disease process through exposure to the native, fully virulent form of the pathogen. We all recognize that vaccines have changed the course of history and that we have benefited enormously from their use as powerful means of protecting against certain highly contagious and dangerous infectious diseases (Figure 17-1). Indeed, one of the greatest achievements of the past century was the complete eradication of smallpox through a massive worldwide campaign conducted in the 1960s and 1970s by the World Health Organization (WHO). After the last known natural case was reported in 1977 in Somalia, the WHO officially declared smallpox eradicated from the world in 1980.

Vaccines are often the most cost-effective medical intervention to prevent infectious disease and death. They are not only much cheaper than the cost of treatment of infectious diseases, but they also reduce suffering and long-term consequences of the disease. Patients who are diagnosed and treated for an infection that is underway have already suffered symptoms serious enough to cause them to seek medical attention, and may even have sustained irreversible damage. In contrast, vaccines prevent the infection from becoming established in the first place, thereby avoiding significant disease symptoms and damage. Importantly, in cases where there is no effective therapy for an infection, vaccines may be the only way to protect patients from serious disease.

Vaccines are clearly a major health care bargain. But, if vaccines are so important, then why are there not more of them? Unfortunately, many of the barriers to vaccine development are financial, legal, or political, rather than scientific. In the market economies of developed countries, there have been financial disincentives to develop or produce new vaccines, especially vaccines that would have their greatest impact in the developing world. Vaccines are not nearly as profitable as cholesterol-lowering drugs or blood pressure medications that have to be taken daily for long periods of time or throughout an individual's life.

In recent years, development and testing of vaccines have shifted to serious diseases that are widespread in a popula-

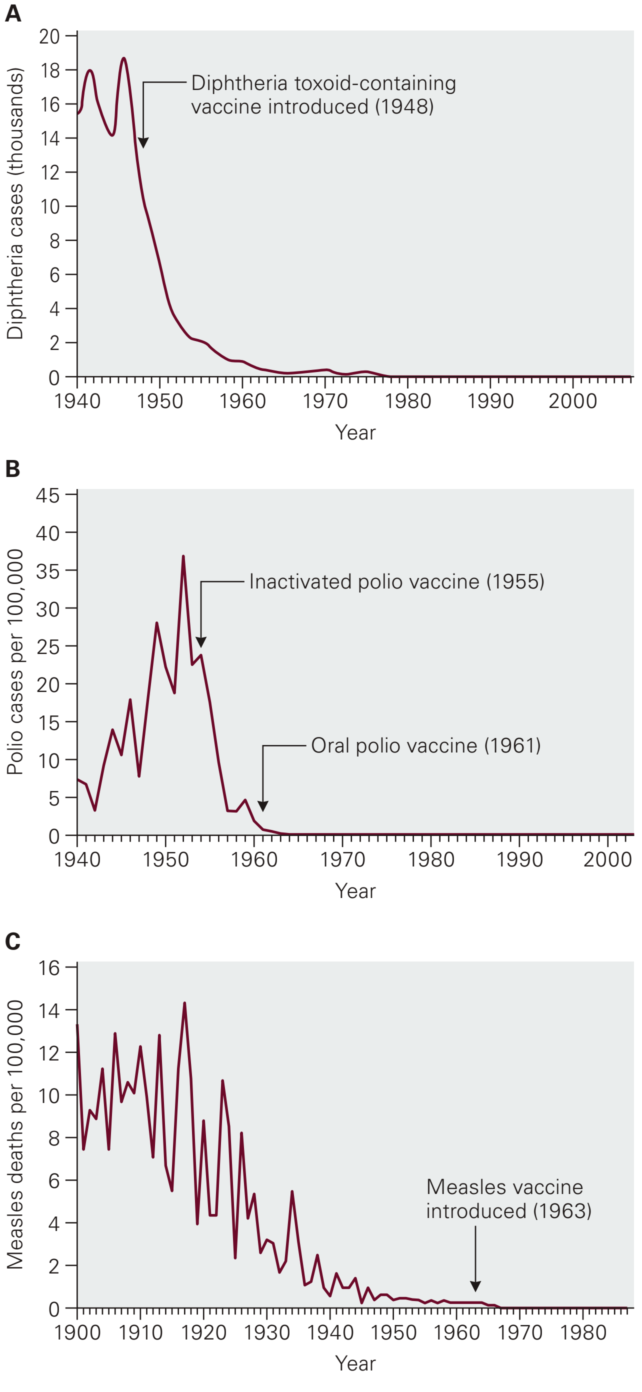


Figure 17-1. Timelines and impacts of major vaccine introductions on disease outbreaks. The introduction of vaccines through immunization programs over the past five decades has played a major role in eliminating certain deadly diseases from the United States. **(A)** The effect of introduction of the diphtheria toxoid–containing vaccine in 1948 on the annual number of diphtheria cases. **(B)** The effect of introduction of the inactivated polio vaccine in 1955 and the attenuated live vaccine in 1961 on the number of polio cases. **(C)** The effect of introduction of vaccine against measles in 1963 on annual deaths due to measles. The mumps component was added in 1967 and the rubella component in 1969 to generate the current trivalent MMR vaccine.

tion, such as otitis media (middle ear infection) or meningitis in children. These newer vaccines, such as the *Haemophilus influenzae* type b (Hib) vaccine, have made important recent contributions in controlling a number of childhood illnesses, at least in developed countries, and some of them have even proven relatively profitable for pharmaceutical companies to produce. Efforts are under way by philanthropic and international health organizations to make these vaccines available worldwide.

What Makes an Ideal Vaccine?

Early work toward developing vaccines was largely based on empirical data and a lot of trial and error. Exciting new advances in next-generation vaccines have now been made possible through our improved understanding of microbial and host factors required for virulence and the nature of the immune response to specific infections. One of the key advances has been in the area of innovative approaches toward enhancing immunogenicity and eliciting the appropriate protective immune response, particularly with regard to stimulating long-lasting cell-mediated immunity. Since vaccines are meant to prepare or prime the immune system for rapid response upon subsequent exposure to the native pathogen, it is important to consider what the requirements of an "ideal" vaccine might be. Table 17-1 summarizes some of the key attributes that modern vaccinologists strive for when designing and developing an effective vaccine. They start by asking a battery of questions that must be addressed in order to carry out their task.

Ideal vaccines should provide long-term protection against infections by stimulating long-lived effector and memory T cells and B cells (see chapter 4). The first two questions that must be considered are where the body first encounters the pathogen and what other parts of the body the pathogen encounters during progression of the infection. Answering these questions will determine what type of immune response will be most effective at preventing infection: mucosal (IgA) versus systemic (IgM, IgG) or both (mucosal at initial site of entry, then systemic once the mucosal defenses have been breached). Additionally, knowing where the infection is taking place further informs us of where to administer the vaccine so as to best elicit an immune response that is protective.

The next questions that must be considered are whether the pathogen is intracellular or extracellular, what virulence factors the pathogen produces, and where they act. Are there any toxins produced? Does the pathogen have a capsule or pili? Does the pathogen have components on its surface that mimic the host or otherwise help it hide from the host? Answering these questions will determine whether a humoral (antibody, Th2) response or cell-mediated (CTL, Th1) response will be most effective and whether a single antigen or multiple antigens will need to be targeted (see chapter 4).

Table 17-1. Ideal vaccine requirements

FACTORS THAT MUST BE TAKEN INTO CONSIDERATION WHEN DEVELOPING A CANDIDATE VACCINE
• An ideal vaccine must be safe with minimal adverse side effects.
• An ideal vaccine must be efficacious—able to elicit a strong and long-lasting protective immune response.
• An ideal vaccine must be readily delivered in combination with other vaccine components. (Nobody likes shots!)
• When targeted against a mucosal pathogen, an ideal vaccine must be effective when administered orally or intranasally to elicit a mucosal immune response.
• An ideal vaccine must be heat-stable, dry-stable, and have a long shelf life.
• An ideal vaccine must be low-cost and easy to manufacture and administer with minimal infrastructural requirements.
• An ideal vaccine requires minimal maintenance programs—the fewer number of boosters required increases the likelihood of compliance to the immunization regimen.

Once virulence factors have been identified as potential vaccine antigens, it is necessary to determine the extent of antigenic variation that might be found in these virulence factors. How rapidly can the pathogen mutate or change its surface components such that the vaccine might lose its efficacy quickly? Choosing an antigen that is highly conserved and triggers an appropriate immune response will be long-lasting and protective. Importantly, to generate an ideal vaccine, it is necessary to determine how best to present the antigens to the immune system for an optimal strong protective response. For this, it is necessary to determine which epitope(s) of an antigen are protective and which will elicit the desired immune response, such as Th1 versus Th2. Recall that Th1 responses are effective against intracellular pathogens, whereas Th2 responses are best at controlling extracellular pathogens (see chapter 4).

It is important to bear in mind that not all **antigens**, defined as a substance that interacts with an antibody, are capable of eliciting an immune response. An **immunogen** is an antigen that elicits an immune response, but not all immunogens elicit protective immune responses. The part of the antigen that is recognized by the antibody (**antigenic determinant**) is called an **epitope**. The epitope may be buried or not exposed in the native antigenic substance such that it is inaccessible to the antibody during infection. Thus, not all immunogenic epitopes elicit protective immune responses. Since protection through a humoral response is dependent on the ratio of antibody to antigen, if the antibody titers are too low and there are not enough memory Th2 cells or B cells, then the invading pathogen can overwhelm the immune system, establish infection, and manifest disease symptoms. In such cases, it may be necessary to administer booster shots to keep the antibody titers high for a more rapid response. However, the more booster shots required for full protection, the greater the cost of delivering a vaccine and the more governmental, medical, industrial, societal, and organizational infrastructure that is needed to maintain the immunization program.

A complication that sometimes arises is that the strongest and most protective immunogenic epitope is not against a single linear sequence of an antigen (a **continuous epitope**), but rather against a structural conformation or against multiple segments of the antigen that have come together to form a topological surface (a **discontinuous epitope**) (see Figure 4-2). In such cases, it is very difficult to replicate the epitope in recombinant vaccines without using the antigen's native structure and any harsh manipulation of the antigen during the vaccine formulation or subsequent storage conditions could result in loss of the protective epitope and loss of vaccine efficacy.

Proper vaccine storage and handling practices are critical for maintaining vaccine quality and efficacy. An ideal vaccine must have properties that enable it to be stored for long periods of time under potentially adverse conditions (e.g., extreme temperatures, wet or dry climates). This means that it must be formulated, packaged, and delivered in such a way that it remains heat-stable, dry-stable, and with a long shelf life. But, at the same time, an ideal vaccine must be easy to manufacture, store, and administer. Maintaining these counterposing attributes are especially relevant for vaccines destined for underdeveloped areas of the world, where refrigeration and good storage conditions are often minimal or absent, the climates are extreme, and the resources available to provide appropriate vaccine handling are limited.

Finally, one of the important requirements of an ideal vaccine is that it must be low-cost with minimal infrastructural requirements and minimal maintenance programs necessary for its successful implementation. A vaccine that requires numerous boosters, that cannot be administered in combination or at the same time as other vaccines, that requires injection (as opposed to oral or nasal inoculation), and that requires refrigeration and special handling is not low-cost or easily administered. Moreover, such traits make it less amenable to application in developing countries that lack the resources and facilities to implement and maintain effective immunization programs.

Having a good vaccine is not sufficient to ensure a successful immunization program. Delivering the vaccine, particularly to poorer countries, requires considerable logistical and infrastructural support. A 2015 cost analysis showed that delivery of a full immunization program across 94 low- and middle-income countries that belong to the **Global Alliance for Vaccines and Immunization (GAVI)** would amount to about $62 billion over the 10-year period of 2011 to 2020, with an estimated $51 billion for routine immunization and $11 billion for supplemental immunizations (boosters). Unfortunately, a follow-up cost analysis in 2016 by GAVI indicated that full implementation of the planned immunization programs would result in a total funding shortfall of $7.6 billion over the period of 2016 to 2020. Much of this funding gap appears to be in service-delivery and supply-chain costs and costs associated with introduction of new vaccines. Clearly, additional resources from wealthier nations and organizations as well as new vaccine formulation and delivery strategies will be required to meet future vaccine implementation goals worldwide.

Immunization Programs

Comprehensive and globally accessible vaccine immunization programs are the key to successful disease intervention and eradication, but the process does not happen quickly, and for most diseases complete success has not yet been achieved. The administration of an effective immunization program requires a large national and often international infrastructure to be in place, with the capacity to facilitate coherent planning, coordination, financing, public information, and government-industry cooperation for the proper testing, production, distribution, administration, and monitoring of vaccines and their efficacy. In the United States alone, this process requires the proper coordination and functioning of over 20 federal agencies, not including state departments of health, vaccine and biotechnology companies, professional medical facilities, voluntary organizations, and public and private health care officials. A clear example of what can happen when this complex infrastructure fails on a large scale is exemplified by the diphtheria epidemic that swept through the former Soviet Union following its collapse in the early 1990s (see Box 17-1).

For any given disease that has a good vaccine available, there are two key milestones that must be met for an immunization program to be successful (Figure 17-2). The first is **elimination**, defined as the reduction of new disease cases to zero in a specified geographical area, but where continued intervention measures are necessary to prevent reintroduction of the disease through transmission from external sources or areas where the disease still occurs. The second, and final, is **eradication**, which is defined as the permanent reduction of new disease cases to zero worldwide, with no further interventions needed since there is no longer any chance of disease contraction from anywhere else. Smallpox was one of only a handful of diseases deemed as a good target for elimination and subsequent eradication, be-

Box 17-1.

What Happens When Infrastructure that Maintains an Immunization Program Fails?

After implementation of a universal immunization program in the 1950s, the incidence of diphtheria in the former Soviet Union was dramatically reduced and remained so for decades. However, just a few years after the collapse of the former Soviet Union, a massive reemergence of diphtheria cases swept through the Newly Independent States of the former Soviet Union in the 1990s, before aggressive and large-scale control measures were able bring the epidemic back under control.

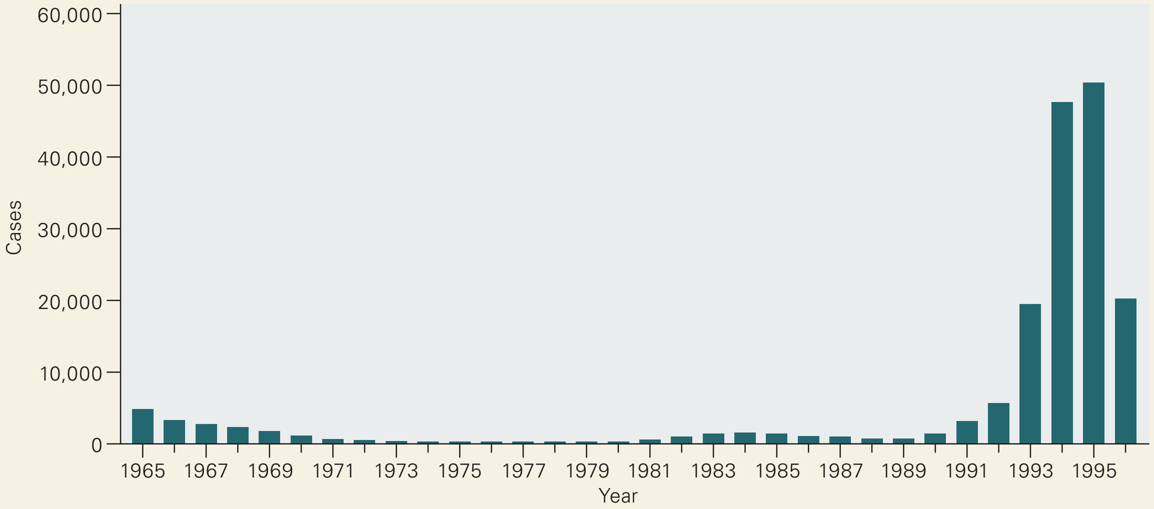
The epidemic began in Moscow in 1990 with exposure to new toxigenic strains of *Corynebacterium diphtheriae*, presumably introduced through return of personnel from military and paramilitary activities in Afghanistan, where diphtheria is endemic. A retrospective examination of the factors that contributed to the rapid spread of the diphtheria epidemic revealed that the socioeconomic instability and large-scale population movements among the regions after the collapse of the Soviet Union significantly increased the susceptibility of both children and adults to diphtheria. This epidemic also illustrates a particular set of circumstances that can shift the age range of a disease. This volatile situation was further undermined by a deteriorated health care infrastructure in most regions that caused shortages of vaccines, antibiotics, and other medical supplies and delays in executing disease containment and control measures. The result was that many adults were not immune and were not protected by the herd immunity conferred by a population of vaccinated children. In some areas the fatality rate was as high for adults as for children. Indeed, the lack of adequate stockpiles of diphtheria antitoxin and delayed treatment resulted in high case fatality rates (>20%) in some of the regions at the beginning of the epidemic, but improved once supply sources for antitoxin and antibiotics were reestablished by the new governments and international assistance in 1995.

Sources:

Vitek CR, Wharton M. 1998. Diphtheria in the former Soviet Union: reemergence of a pandemic disease. *Emerg Infect Dis* **4:**539–550.

Dittmann S, Wharton M, Vitek C, Ciotti M, Galazka A, Guichard S, Hardy I, Kartoglu U, Koyama S, Kreysler J, Martin B, Mercer D, Rønne T, Roure C, Steinglass R, Strebel P, Sutter R, Trostle M. 2000. Successful control of epidemic diphtheria in the states of the Former Union of Soviet Socialist Republics: lessons learned. *J Infect Dis* **181**(Suppl 1)**:**S10–S22.

Vitek CR, Bogatyreva EY, Wharton M. 2000. Diphtheria surveillance and control in the former Soviet Union and the Newly Independent States. *J Infect Dis* **181**(Suppl 1)**:**S23–S26.



Reported diphtheria cases in the former Soviet Union and the Newly Independent States, 1965–1996.

cause it met all of the criteria considered necessary for eradication:

(1) It is a human-specific disease with no known animal reservoir for the pathogen, such that person-to-person transmission can be prevented, with no risk of reintroduction through animal exposure.

(2) There are excellent sensitive and specific diagnostic and surveillance tools available for rapid identification and epidemiologic tracking.

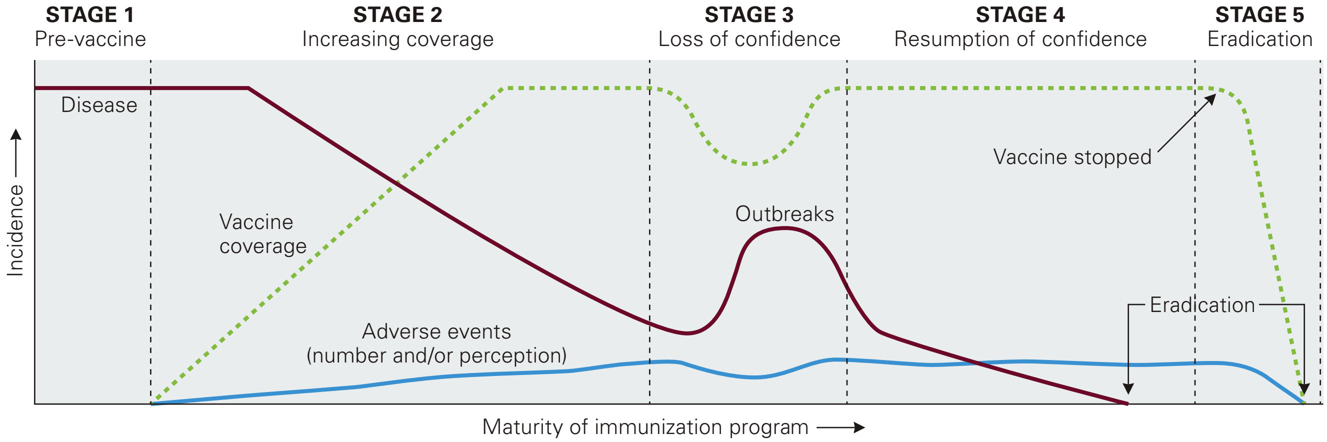


Figure 17-2. The stages of immunization program development. There are five main stages involved in the development of a large-scale immunization program. In stage 1, the disease incidence is high, necessitating formulation and development of a vaccine, as well as setting up the logistics of delivering the vaccine to the public. In stage 2, the vaccine is increasingly being administered, and as a result, the incidence of disease declines. During this stage, any associated adverse reactions or events begin to appear, until a point that public awareness of these adverse events or perception of adverse vaccine-related problems arise. Loss of confidence in the vaccine during stage 3 may result in limited outbreaks of the disease in certain geographical areas, which leads to resumption and reinforcement of the need for vaccination in stage 4. Continued confidence in the vaccine maintains the immunization program until the disease is eliminated from an increasingly large geographic range. Eventually, the disease incidence is completely reduced to zero cases reported anywhere in the world, resulting in the eradication of the disease. At stage 5, the disease is declared eradicated and the immunization program for that vaccine is completed. Adapted from Chen RT, Shimabukuro TT, Martin DB, Zuber PL, Weibel DM, Sturkenboom M. 2015. *Vaccine* **33(Suppl 4):**D46–54, with permission.

(3) Survival from infection or vaccination confers lifelong protective immunity.

(4) There is a high international health care and financial burden associated with the disease, such that commitment to its elimination and eradication will be highly beneficial on a global scale.

Today, worldwide campaigns continue against polio and the other diseases targeted for eradication (yellow fever, malaria, yaws, hepatitis B, rubella, measles, mumps, rabies, guinea worm, and hookworm). However, each of these diseases is proving more challenging to eradicate than smallpox, mostly due to problems associated with not meeting one of the previously mentioned criteria. For example, in the case of measles, mumps, and rubella, all of which have a good trivalent vaccine (**MMR vaccine**) available, the main hindrance to eradication is the political and financial support needed to achieve and maintain the high immunization levels required for these highly infectious diseases.

In the cases of yellow fever virus, rabies, and malaria, there are animal or insect vector reservoirs that pose continual potential for zoonotic transmission. **Reservoirs** are sites (animate or inanimate) in which infectious agents remain viable and from which individuals can acquire infection. Direct host-to-host transmission occurs through direct contact between an infected individual and an uninfected individual. Indirect host-to-host transmission occurs through carriers, in the form of inanimate vehicles (water, food), inanimate fomites (bedding, toys, books, surgical instruments, door handles), or living vectors (insects, animals). Insects (referred to as **arthropod vectors**) may or may not be hosts for pathogens, but they can carry the pathogen from one host to another. For many pathogens, the reservoir host is essential for the life cycle of the infectious agent and requires person-to-person transmission. Pathogens that are dependent on a single host species for transmission are easier to control and eradicate. Some pathogens have both animal and human host reservoirs. A disease that is primarily confined to animal reservoirs is **epizootic**. A disease that primarily infects animals but is occasionally transmitted to humans is **zoonotic**. Eradication of a zoonotic disease from humans can only be successfully accomplished through co-elimination of the disease in the animal reservoir.

Although polio has an excellent oral vaccine and it is thought to be near eradication (99%), there have been some recent setbacks with the disease persisting in a few endemic areas (Afghanistan, Pakistan, and Nigeria). Part of the problem is that since the vaccine is a live, attenuated oral vaccine, maintaining its efficacy in extremely hot and remote areas is difficult. An **endemic** disease is one that is constantly present, usually at low incidence, in a population. Individuals infected with an endemic disease can serve as reservoirs of infection and may be a source from which transmission can occur sporadically. Vulnerable unimmunized individuals who

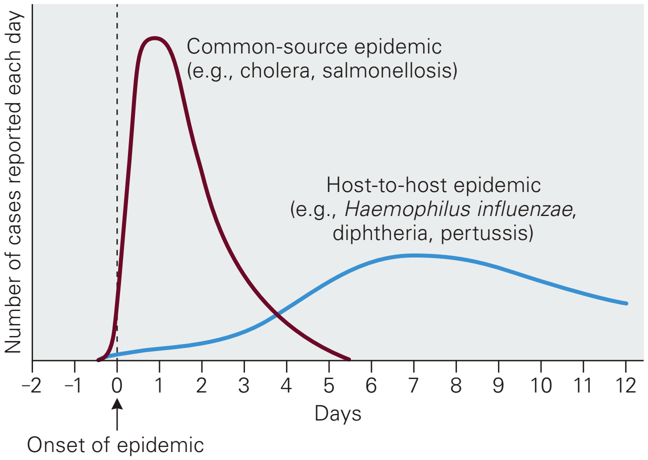


Figure 17-3. Epidemic diseases. There are two types of epidemic diseases. Common-source epidemics arise due to infection (or intoxication) of a large number of people from a common contaminated source, such as water or food. Host-to-host epidemics arise by the introduction of a single individual into a susceptible population, with this individual infecting one or more people.

travel to regions where endemic disease is present are at risk of acquiring the disease and spreading it to others when they return to their homes. In contrast, an **epidemic** disease is one that simultaneously, and often suddenly, infects an unusually high number of individuals in a population. Common-source epidemics are caused by infection of a large number of people from a common contaminated source (e.g., water or food), whereas host-to-host epidemics arise by the introduction of a single individual into a susceptible population, with this individual infecting one or more people (Figure 17-3). Host-to-host epidemics tend to last much longer. Once an epidemic has become widespread, involving multiple countries and continents, it is considered a **pandemic** disease and is much more challenging to control and eradicate.

Barriers to Implementation and Success of Immunization Programs

A different type of vaccine problem, and one that at least in theory should be easier to solve, is the problem of needing a simpler vaccine regimen than the current confusing mixture of multiple and separate inoculations of different vaccines at different times, particularly during childhood. Logistically, it is much less complicated to administer more than one vaccine simultaneously in a single dose, preferably by the oral, skin, or nasal route, than to eliminate the need for so many booster shots. Using live viral or bacterial vaccine strains that present antigens of more than one pathogen is another possible solution.

The ability to administer several different vaccines in a single swallow, a single inhalation, or even a single shot has obvious appeal. One of these types of combination vaccine, first introduced in 1949, was the DTP (also known as the DTwP) vaccine, which combined component vaccines against diphtheria, tetanus, and pertussis. The diphtheria and tetanus antigens in the DTP vaccine are the detoxified forms of the bacterial protein toxins (called **toxoids**). The DTP (or DTwP) vaccine was comprised of diphtheria toxoid (D), tetanus toxoid (T), and heat- or chemically inactivated whole-cell extracts of the *Bordetella pertussis* pathogen (wP). For safety reasons, due to adverse reactions caused by the whole-cell pertussis component, this vaccine was replaced in the 1990s by the DTaP and Tdap vaccines, which are comprised of diphtheria toxoid, tetanus toxoid, and up to three pertussis antigens: pertussis toxoid, pertactin, and filamentous hemagglutinin antigen. The lowercase “d” and “p” in Tdap, which is the vaccine currently recommended by the CDC for booster shots, indicate a lower dose of the diphtheria and pertussis toxoids than that used in the DTaP vaccine, and the “a” refers to the use of “acellular” pertussis antigens. The U.S. Food and Drug Administration (FDA) approved an even larger multicomponent vaccine, called Pediarix, in 2002. Pediarix combines three different existing vaccines into one shot: (1) the components of DTaP, (2) hepatitis B virus (HBV) surface antigen, and (3) all three chemically inactivated poliovirus variants. Use of Pediarix reduces the number of shots in the first 6 months of childhood from 10 to 3.

Just as we saw for antibiotics in chapter 15, industry has generally shied away from the task of developing new vaccines. The long, arduous, and costly process of getting a vaccine developed and approved for market is very similar to that encountered for antibiotics (see Figure 15-4). In addition, there have been legal reasons for industry’s general lack of enthusiasm for vaccine development. Since most vaccines are mainly used in young children, the degree of scrutiny regarding safety is exceedingly high. Industry has been discouraged by the numerous lawsuits based on alleged rare side effects of vaccines, which can be quite expensive for companies, especially when they involve children. Juries also may not be adequately informed of the fact that many of the alleged side effects of vaccines are not supported by scientific studies. An unexpected **adverse event** following immunization does not necessarily have a direct causal relationship with the vaccine itself, as discussed in the next paragraph. While some adverse events may be due to the vaccine, some can occur through improper administration of the vaccine or may be the result of an entirely unrelated coincidence.

Those vaccines that have been used in national immunization programs have proven to be extremely safe and effective, but there is no question that each new vaccine, like every medication, carries a risk that rare, sometimes serious, adverse side effects may occur. Most side effects from a vaccine are mild, usually soreness, swelling, or redness at the injection site, with some vaccines causing mild fever, rash, or muscle aches. Occasionally, more serious adverse side effects are encountered, including death. Public trust in vaccine safety is key to the success of any immunization program. In an era where most people in developed countries have not experienced the horrors of an outbreak of smallpox, polio, whooping cough, or diphtheria; have not watched a child die of bacterial meningitis; or have not witnessed the anguish of a mother who gives birth to a malformed child because she contracted rubella during pregnancy; it is easy to take these benefits for granted and to focus instead on the rare side effects that are the inevitable cost of such benefits.

There are also formidable technical barriers to the development of vaccines against some of the diseases considered most important today: AIDS, malaria, gonorrhea, tuberculosis, and pneumonia. To understand some of the technical problems that have frustrated development of vaccines against these infectious diseases, it is first useful to understand how successful vaccines work, because successful vaccines illustrate traits of infectious microbes and viruses that make them amenable to vaccine development and its subsequent implementation through an immunization program (see Table 17-1). Pathogens that do not have these traits pose a much greater vaccine challenge. It is also encouraging to recall that despite setbacks in recent years, there have been some highly effective vaccines developed in the not-so-distant past. Moreover, several nonprofit research organizations funded by private charitable foundations are spearheading the use and development of cost-effective vaccines in developing countries.

The Antivaccination Movement

There is a small but vocal group of individuals who refuse to have their children vaccinated. These individuals are now working to make refusal of vaccinations possible for people who cannot claim religious grounds for their objections. Why would parents willingly expose their children to such horrible diseases as diphtheria and whooping cough, which used to kill many thousands of children? It certainly is not because these diseases have disappeared. If you need to be convinced that these diseases are still around, consider the "involunteer experiment" done accidentally in the United Kingdom during the 1970s and 1980s. In response to sensational and highly inaccurate news accounts of alleged serious side effects of the vaccine against whooping cough, parents of young children began to refuse to have them receive the DTP (also known as the DTwP) vaccine, and vaccination rates dropped from around 80% to 30% (Figure 17-4). Instead of the three-component DTP vaccine routinely administered to infants and toddlers at that time, parents demanded—and their children received instead—a DT formulation that lacked the pertussis component (wP) of the vaccine. The effects of this miscalculation by the parents were tragic, but unfortunately entirely predictable. A series of epidemics of whooping cough subsequently occurred in the United Kingdom, in which about 100,000 children were infected and 36 died. Those children who survived went through a horrible experience. The disease produces a cough so violent and repetitive that children feel as if they are suffocating. Permanent brain damage occurred in some of those who survived.

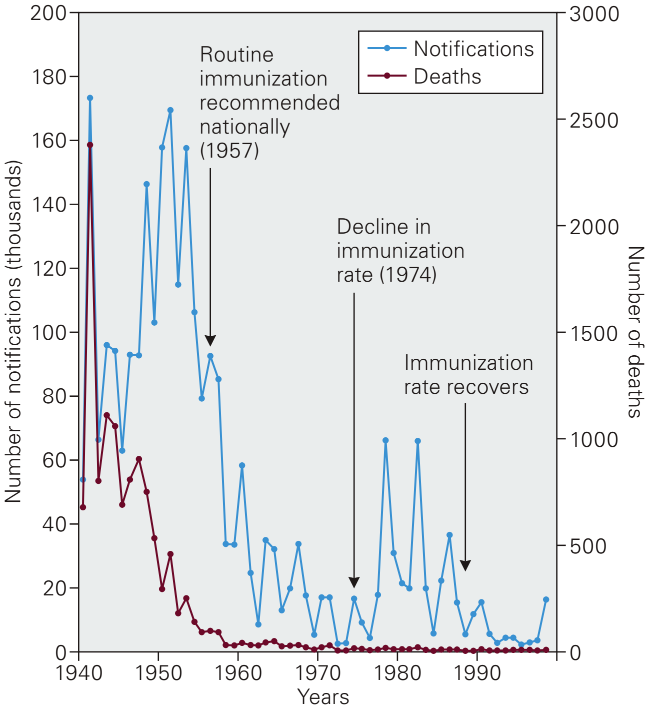


Figure 17-4. Example of the impact of the antivaccination movement. Public backlash against the pertussis vaccine resulted in a large drop from around 80% to 30% in vaccination rates in the United Kingdom during the mid-1970s, which in turn was followed by a series of whooping cough epidemics. Adapted from Baker JP. 2003. *Vaccine* **21**:4003–4010, with permission from Elsevier.

Given this, why do parents refuse to have a child vaccinated? Most of these parents were well intentioned and believed allegations that vaccines cause all manner of evils, particularly neurological damage. The reason for this association is easy to understand. Most neurological problems in children appear in the first few years of life, the same time as when virtually all children in developed countries are receiving their vaccinations. But, as we stressed *ad tedium* in chapter 6, association is not proof of cause and effect. In particular, in cases where over 80% of the population experiences a particular treatment, that treatment could be associated with almost anything. And, as for the argument that there are fewer cases of neurological disorders in developing countries where vaccinations are not available, the tragic fact is that most children in such countries who develop neurological problems do not live long enough to be counted.

In an attempt to introduce some medical rationality into the debate over vaccine safety, the Institute of Medicine, an independent research agency sponsored by the National Academy of Sciences, undertook a painstaking review of the purported side effects of vaccines in 1994. Their conclusions, summarized in Table 17-2, were that there is little or no evidence for most of the alleged side effects of vaccines, and where there might be a connection, the side effects are rare and not of the most serious type. This and other studies since have also emphasized the benefit statistics, which are almost never included in sensational press articles about vaccines. For vaccines recommended in the United States, the reduction in incidence of the targeted disease is at least 96%, and in most cases 99% or higher (Table 17-3). Reductions of nearly 100% in the case of diseases such as polio have made it possible to attempt to eradicate the disease. This has already been accomplished with smallpox, and if it were not for the bioterrorists eyeing the few remaining stocks of smallpox virus in scientists' freezers, we could safely forget smallpox, a disease that terrorized Europe, scourged Asia, and wiped out whole populations of Native Americans.

Those who refuse to have their children vaccinated are—quite consciously in many cases—taking advantage of a phenomenon known as **herd immunity**. Herd immunity arises from the fact that an unvaccinated person in a population that is mostly vaccinated will be protected from disease, because there are not enough susceptible people in this population to allow infectious disease transmission and an outbreak to materialize (Figure 17-5). Based on studies from outbreaks of moderately infectious diseases, such as polio and diphtheria, if at least 70% of the population is immunized, then the rest will be protected. However, for highly infectious diseases, such as influenza, mumps, and measles, up to 95% of the population must be immunized to confer herd immunity.

There is a serious flaw in relying on herd immunity, however, given the increase in international travel. Children who have not been immunized against viral diseases, such as measles and mumps, and who have been protected from measles and mumps during childhood years due to the herd effect, would be ill advised to travel during their adult years to areas of the world where measles is still endemic, as noted previously. For reasons that are still not well understood, many of these diseases have a much more serious effect if they strike teens or young adults than if they occur in children. Parents who refuse to have their children vaccinated are setting those children up for a dangerous future.

Vaccine Success Stories

Vaccines against smallpox, a viral disease, and diphtheria, a bacterial disease, have virtually eradicated these diseases in countries with vaccination programs in effect. A good vaccine can provide lifelong immunity to an infectious disease by stimulating long-lived effector and memory T cells and B cells, as described in chapter 4. Some of the vaccine success stories are summarized in Table 17-3. We will now focus on a few of those successful vaccines that are specific for bacterial diseases.

Subunit Vaccines

Most successful vaccines used today against bacterial pathogens work by inducing humoral immunity against bacterial antigens that are relatively invariant (e.g., secreted toxins or surface proteins). A great example of this is the trivalent DTaP vaccine used to protect against diphtheria, tetanus, and whooping cough. These diseases are caused by protein toxins released into the bloodstream by invading bacteria. An effective way of counteracting these circulating toxins is through the production of neutralizing antibodies. The DTaP vaccine works by stimulating the production of these antibodies. Vaccines like DTaP are called **subunit vaccines** because they consist of one or more purified proteins that have been inactivated through heat or chemical treatment. The inactivated forms of the toxin proteins or their subunits are referred to as toxoids. These toxoid-based vaccines are administered extracellularly, and so they primarily elicit a humoral antibody response rather than a cell-mediated response (see chapter 4). Vaccines based on synthetic epitope-containing fragments of toxins are considered later in this chapter. So long as high titers of these antibodies are maintained in the body, even if the bacteria producing these toxins colonize a person, no disease symptoms will develop because the circulating antibodies neutralize the toxins. This property makes toxin-producing bacteria among the easiest and most efficacious vaccine targets.

Diphtheria and tetanus toxoids can be administered to infants, and side effects are rare. This is not true of the wP part of the old DTwP vaccine. Although the wP vaccine was very successful in preventing whooping cough, a disease that was once a common killer of children, it had some troublesome side effects. In about 20% of infants given DTwP, side effects ranged from generalized discomfort, which made the infant fussy for a couple of days, to convulsions. The wP vaccine sometimes also caused hearing loss or brain damage in some children, although this side effect was extremely rare. The reason the wP vaccine had so many side effects compared to the diphtheria and tetanus toxoids is that it consisted of whole killed *B. pertussis* bacterial cells, not just the pertussis toxin toxoid, and thus it was a complex mixture of antigens, some of which caused adverse effects.

New, more defined versions of the pertussis vaccine, called acellular pertussis (aP) vaccines, are now available, which usually consist of the pertussis toxoid plus one or more *B. pertussis* cell surface components (e.g., **filamentous hemagglutinin [FHA]**, a 69-kilodalton outer-membrane protein

Table 17-2. **Summary of conclusions based on evidence regarding the possible association between specific adverse effects and receipt of childhood vaccines, by determination of causality—Institute of Medicine (IOM), 1994[a]**

DT/Td TOXOID[b]	MEASLES VACCINE[c]	MUMPS VACCINE[c]	OPV/IPV[d]	HEPATITIS B VACCINE (HBV)	*HAEMOPHILUS INFLUENZAE* TYPE B (HIB) VACCINE
1. No evidence was available to establish a causal relationship					
None	None	Neuropathy Residual seizure disorder	Transverse myelitis (IPC) Thrombocytopenia (IPV) Anaphylaxis (IPV)	None	None
2. Inadequate evidence to accept or reject a causal relationship					
Residual seizure disorder other than infantile spasms Demyelinating diseases of the central nervous system Mononeuropathy Arthritis Erythema multiforme	Encephalopathy Subacute sclerosing panencephalitis Residual seizure disorder Sensorineural deafness (MMR) Optic neuritis Transverse myelitis Guillain-Barré syndrome Thrombocytopenia Insulin-dependent diabetes mellitus	Encephalopathy Aseptic meningitis Sensorineural deafness (MMR) Insulin-dependent diabetes mellitus Sterility Thrombocytopenia Anaphylaxis[e]	Transverse myelitis (OPV) Guillain-Barré syndrome (IPV) Death from SIDS[f]	Guillain-Barré syndrome Demyelinating diseases of the central nervous system Arthritis Death from SIDS[f]	Guillain-Barré syndrome Transverse myelitis Thrombocytopenia Anaphylaxis Death from SIDS[f]
3. Evidence favored rejection of a causal relationship					
Encephalopathy[g] Infantile spasms (DT only)[h] Death from SIDS (DT only)[h,i]	None	None	None	None	Early-onset Hib disease (conjugate vaccines)
4. Evidence favored acceptance of causal relationship					
Guillain-Barré syndrome[j,k] Brachial neuritis[j]	Anaphylaxis[e]	None	Guillain-Barré syndrome (OPV)[k]	None	Early-onset Hib disease in children aged >18 mo whose first Hib vaccination was with unconjugated vaccine
5. Evidence established a causal relationship					
Anaphylaxis[j]	Thrombocytopenia (MMR) Anaphylaxis (MMR)[e] Death from measles vaccine strain viral infection[f,l]	None	Poliomyelitis in recipient or contact (OPV) Death from measles vaccine strain viral infection[f,l]	Anaphylaxis	None

[a] Reprinted from CDC. 1996. Update: vaccine side effects, adverse reactions, contraindications, and precautions—recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Morb. Mortal. Wkly. Rep.* **45**(RR-12):1–35. This table is an adaptation of a table published previously by the Institute of Medicine (IOM) (K. R. Stratton, C. J. Howe, and R. B. Johnston [ed.]. 1994. *Adverse Events Associated with Childhood Vaccines: Evidence Bearing on Causality*. Institute of Medicine, National Academy Press, Washington, DC). IOM is an independent research organization chartered by the National Academy of Sciences. The National Childhood Vaccine Injury Act of 1986 mandated that IOM review scientific and other evidence (e.g., epidemiologic studies, case series, individual case reports, and testimonials) regarding the possible adverse consequences of vaccines administered to children. IOM constituted an expert committee to review and summarize all available information; this committee created five categories of causality to describe the relationships between vaccines and specific adverse events.

[b] DT, diphtheria and tetanus toxoids for pediatric use; Td, tetanus and diphtheria toxoids for adult use.

[c] If the data derived from studies of a monovalent preparation, then the causal relationship also extended to multivalent preparations. If the data derived exclusively from studies of the measles-mumps-rubella (MMR) vaccine, the vaccine is specified parenthetically. In the absence of data concerning the monovalent preparation, the causal relationship determined for the multivalent preparations did not extend to the monovalent components.

[d] For some adverse events, the IOM committee was charged with assessing the causal relationship between the adverse event and only oral poliovirus vaccine (OPV) (i.e., for poliomyelitis) or only inactivated poliovirus vaccine (IPV) (i.e., for anaphylaxis and thrombocytopenia). If the conclusions for the two vaccines differed for the other adverse events, the vaccine to which the adverse event applied is specified parenthetically.

[e] The evidence used to establish a causal relationship for anaphylaxis applies to MMR vaccine. The evidence regarding either monovalent measles vaccine favored acceptance of a causal relationship, but this evidence was less convincing than that for MMR vaccine because of either incomplete documentation of symptoms or the possible attenuation of symptoms by medical intervention.

[f] This table lists weight-of-evidence determinations only for deaths that were classified as sudden infant death syndrome (SIDS) and deaths that were a consequence of vaccine strain viral infection. However, if the evidence favored the acceptance of (or established) a causal relationship between the vaccine and a possibly fatal adverse event, then the evidence also favored the acceptance of (or established) a causal relationship between the vaccine and death from the adverse event. Direct evidence regarding the death in association with a vaccine-associated adverse event was limited to (i) Td and Guillain-Barré syndrome, (ii) tetanus toxoid and anaphylaxis, and (iii) OPV and poliomyelitis.

[g] The evidence derived from studies of DT. If the evidence favored rejection of a causal relationship between DT and encephalopathy, then the evidence also favored rejection of a causal relationship between Td and tetanus toxoid encephalopathy.

[h] Infantile spasms and SIDS occur only in an age group that is administered DT but not Td or tetanus toxoid.

[i] The evidence derived primarily from studies of DTP, although the evidence also favored rejection of a causal relationship between DT and SIDS.

[j] The evidence derived from studies of tetanus toxoid. If the evidence favored acceptance of (or established) a causal relationship between tetanus toxoid and an adverse event, then the evidence also favored acceptance of (or established) a causal relationship between DT and Td and the adverse event.

[k] This conclusion differs from the information contained in the Advisory Committee on Immunization Practices recommendations because of new information that became available after IOM published this table.

[l] Deaths occurred primarily among persons known to be immunocompromised.

Table 17-3. Baseline 20th century annual morbidity (cases of disease) before the vaccine became available and 1998 morbidity from nine diseases with vaccines recommended before 1990 for universal use in children[a]

DISEASE	BASELINE 20TH CENTURY ANNUAL MORBIDITY	1998 MOBIDITY	% DECREASE
Smallpox	48,164[b]	0	100
Diphtheria	175,885[c]	1	100[d]
Pertussis	147,271[e]	6,279	95.7
Tetanus	1,314[f]	34	97.4
Poliomyelitis (paralytic)	16,316[g]	0[h]	100
Measles	503,282[i]	89	100
Mumps	152,209[j]	606	99.6
Rubella	47,745[k]	345	99.3
Congenital rubella syndrome	823[l]	5	99.4
Haemophilus influenzae type b	20,000[m]	54[n]	99.7

[a] Reprinted from *MMWR Morb. Mortal. Wkly. Rep.* **48**:243–247, 1999.

[b] Average annual number of cases during 1900 to 1904.

[c] Average annual number of reported cases during 1920 to 1922, 3 years before vaccine development.

[d] Rounded to nearest tenth.

[e] Average annual number of reported cases during 1922 to 1925, 4 years before vaccine development.

[f] Estimated number of cases based on reported number of deaths during 1922 to 1926, assuming a case-fatality rate of 90%.

[g] Average annual number of reported cases during 1951 to 1954, 4 years before vaccine licensure.

[h] Excludes one case of vaccine-associated polio reported in 1998.

[i] Average annual number of reported cases during 1958 to 1962, 5 years before vaccine licensure.

[j] Number of reported cases in 1968, the first year reporting began and the first year after vaccine licensure.

[k] Average annual number of reported cases during 1966 to 1968, 3 years before vaccine licensure.

[l] Estimated number of cases based on seroprevalence data in the population and on the risk that women infected during a childbearing year would have a fetus with congenital rubella syndrome.

[m] Estimated number of cases from population-based surveillance studies before vaccine licensure in 1985.

[n] Excludes 71 cases of *Haemophilus influenzae* disease of unknown serotype.

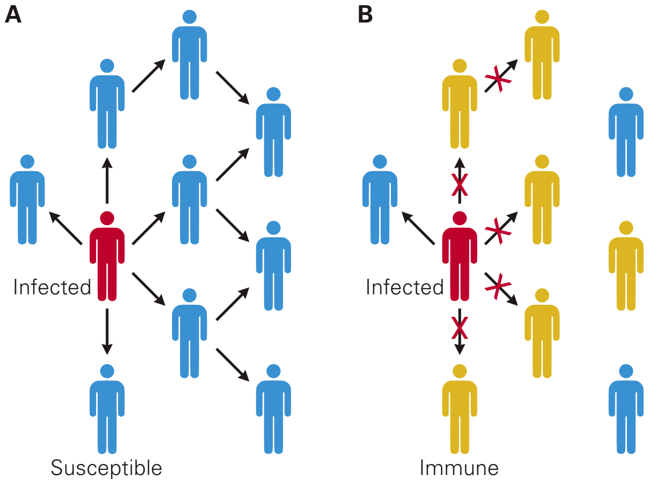


Figure 17-5. Herd immunity. If a high proportion of individuals in a population are immune to a pathogen, then the whole population will be protected from infection. The more highly infectious a pathogen is or the longer its period of infectivity lasts, then the greater the proportion of immune individuals in that population must be to confer herd immunity. Arrows show the normal direction of disease transmission within a population from infected individual to susceptible or immune individual. Adapted from *Brock Biology of Microorganisms* 11th Ed, 2006, with permission.

called **pertactin [Pn]**, and/or **fimbriae [Fim]** types 2 or 3). The new aP vaccines have fewer side effects than the wP vaccine, although their efficacy is somewhat reduced compared to the whole-cell vaccine. Indeed, there has been a recent upsurge in the number of whooping cough outbreaks around the world. Application of next-generation whole genome sequencing technologies to compare clinical isolates from around the world and from different time periods has yielded insights into possible reasons for this trend. Results point to a potential role for vaccine introduction in shaping the evolution of *B. pertussis* as a pathogen. Comparison of allele frequencies indicates that vaccination creates strong selection pressure that reduces the diversity in the pathogen population, but certain allelic variants or "escape mutations" of antigens used in the vaccine enable clonal expansion and clinical circulation of *B. pertussis* strains containing those alleles to escape from the humoral immune response elicited by the aP vaccine (Figure 17-6).

The pertussis vaccine story illustrates the general principle that the simplest vaccines, which consist only of one or a few well-defined antigens, are the ones least likely to cause side effects. In theory, the simple vaccines should also make it easier to target the desirable type of immune response. As

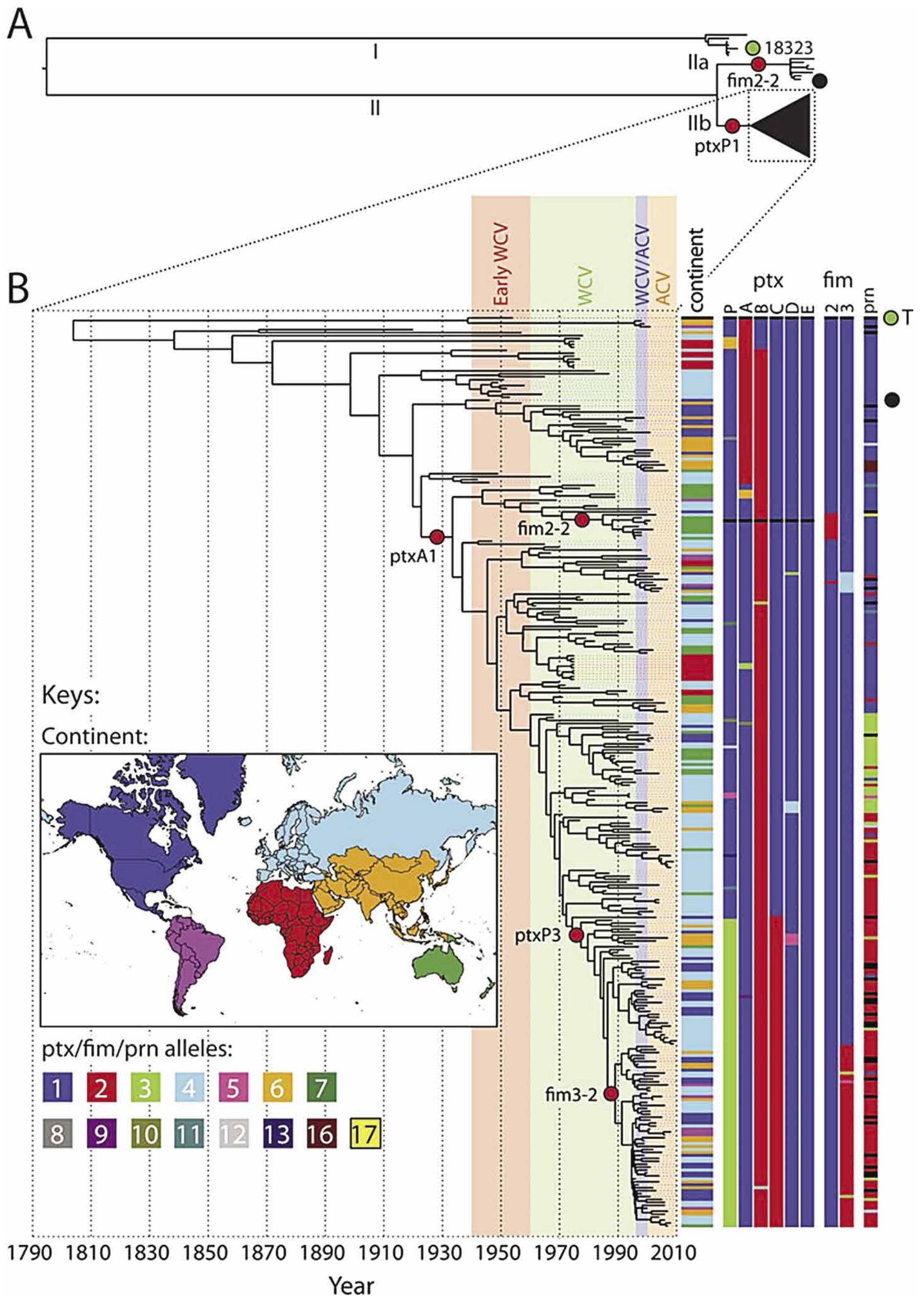


Figure 17-6. Impact of vaccination programs on global phylogeny of *Bordetella pertussis*. Comparative whole genome analysis of a total of 343 *Bordetella pertussis* strains isolated worldwide over many decades using next-generation sequencing technologies provided insights into the diversity and evolution of *B. pertussis* over recent history and the impact that vaccination programs have had. **(A)** Shown is a phylogenetic tree of genomes sequenced, indicating deep divergence between lineages I and II that occurred about 2,000 years ago. Lineage I represents a clade that does not appear to be observed any longer. Most recent isolates are in Lineage IIb, which emerged before introduction of vaccination, but appear to have undergone several recent diversification and expansion events coinciding with introduction of first the whole-cell vaccines (WCV) and then acellular vaccines (ACV). **(B)** Shown is a more detailed phylogenetic tree of isolates within the most common recent clade of *B. pertussis*. Reconstructed dates are shown along the *x* axis of the tree. Dates of vaccination periods are indicated as background colors behind the tree. To the right of the tree, the continent of origin of isolates is indicated by the first column of horizontal bars, colored according to the map in the inset key. The remaining nine columns represent loci within the pertussis toxin *ptx* operon, the fimbrae *fim2* and *fim3* loci, and the pertactin *prn* locus, with assigned numerical alleles colored according to the key. In panels A and B, green-filled circles represent the positions of two reference *B. pertussis* strains 18323 and Tohama I (T) and black-filled circles represent the American vaccine strains B308 (A) and B310 (B). Red circles indicate the major changes in antigen gene alleles in proteins used in current ACVs (from *ptxA2* to *ptxA1*, *fim2-1* to *fim2-2*, *ptxP1* to *ptxP3*, and *fim3-1* to *fim3-2*). Adapted from Bart MJ, Harris SR, Advani A, Arakawa Y, Bottero D, Bouchez V, Cassiday PK, Chiang CS, Dalby T, Fry NK, Gaillard ME, van Gent M, Guiso N, Hallander HO, Harvill ET, He Q, van der Heide HG, Heuvelman K, Hozbor DF, Kamachi K, et al. 2014. *mBio* **5:**e01074.

already mentioned in the case of the DT vaccine, subunit vaccines elicit an antibody rather than a cell-mediated response. Whooping cough is a more complex disease than tetanus or diphtheria, with more than one virulence factor responsible for disease symptoms. Since *B. pertussis* replicates extracellularly in the lung airways, a cell-mediated response is not essential in this case, as it would be if the bacteria multiplied intracellularly. However, both antibodies that neutralize the pertussis toxin and antibodies that bind to surface antigens to facilitate opsonization of the bacteria are needed to control the infection, hence their inclusion in the aP vaccine.

The DTaP vaccine also shares a common problem with other subunit vaccines. Prolonged exposure to antigens is needed to generate a good memory T and B cell response. Since subunit vaccine components do not replicate in the body, they must be administered repeatedly to elicit a maximally effective memory response. The DTaP vaccine has to be administered in a series of five injections, one initial inoculation plus four boosters (Figure 17-7). Although the vaccine regimen produces very long-term immunity to diphtheria and tetanus, a tetanus booster is usually administered again to persons with the sort of wounds associated with tetanus, to ensure that antibody titers are high enough to be protective.

The requirement for boosters increases the likelihood that a child will not receive the full set of vaccinations before being exposed to a disease and will thus not be adequately protected. The problem of children who do not receive the fully recommended course of vaccine administration is still a formidable one even in developed countries, but particularly for developing countries (see Box 17-2). Workarounds for this need to have multiple boosters are currently under investigation and will be discussed later in this chapter. These include having the vaccine antigens produced by a self-replicating microbe; exogenously expressing and secreting the antigens in the host cell using DNA vaccines; or encapsulating the antigens so that they are released slowly over time to provide prolonged exposure.

Conjugate Vaccines

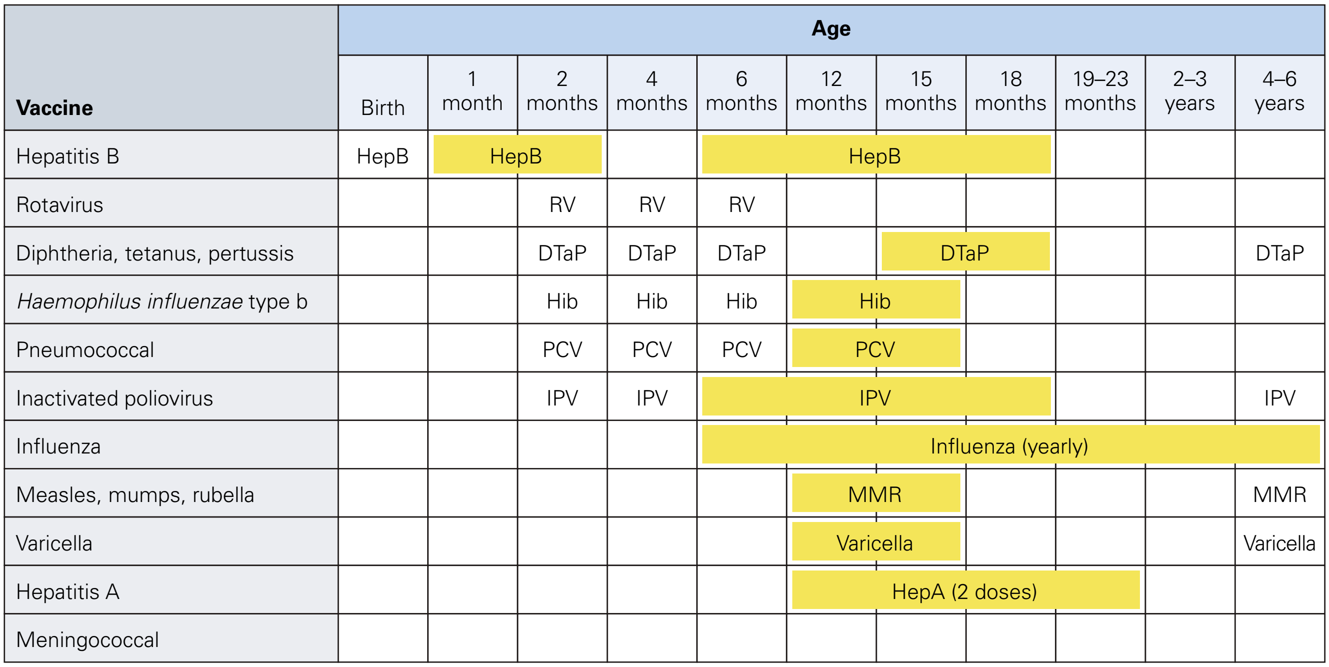
Another successful antibacterial vaccine is the one that protects against childhood meningitis caused by the Gram-negative bacterium *H̲aemophilus i̲nfluenzae* type b̲ (hence the vaccine name, **Hib**). *H. influenzae* type b used to be the most common cause of fatal meningitis in children and also caused epiglottitis, a rapidly progressing disease that can close the airways and lead to suffocation. *H. influenzae* type b also causes pneumonia, particularly in the elderly.

Unlike the bacteria that cause diphtheria, tetanus, and whooping cough, *H. influenzae* does not produce a major protein toxin. Instead, *H. influenzae* coats its surface with a polysaccharide capsule that prevents C3b binding, which allows the bacteria to avoid phagocytosis and clearance. Uncontrolled growth of the bacteria triggers an inflammatory response that results in local tissue damage and can spread and develop into septic shock. Inflammation of the lining that separates the brain and spinal cord from the rest of the body (the meninges) allows the bacteria and blood components to enter spinal fluid, where cytokines released by macrophages and other cells cause increased pressure on the brain that can lead to brain damage. Antibodies that bind to capsular polysaccharides allow phagocytes to opsonize and kill the bacteria, and thus prevent this destructive (and frequently fatal) process from occurring.

Since *H. influenzae* causes meningitis primarily in children under the age of 5 years, it is necessary to immunize young children as early as possible. The problem encountered in developing an effective vaccine was that capsular polysaccharides, which are recognized primarily through T cell-independent mechanisms, do not reliably elicit protective, long-lasting antibody responses (see chapter 4). In addition, young children do not mount effective T cell–independent antibody responses to polysaccharides. To solve these problems, the *H. influenzae* type b capsular polysaccharide antigens were attached covalently to a protein antigen to produce a **conjugate vaccine**. This way, the immune system processes the vaccine antigen through the MHC II pathway, as if it were a protein, producing a strong Th cell-dependent response that leads to long-lasting humoral immunity against the protein antigen and the polysaccharide capsule. There are currently three different proteins that are used for conjugation, all of which are highly effective and safe: inactivated tetanus toxoid, diphtheria toxoid, and meningococcal group B outer membrane protein. These vaccines have been highly effective, dramatically reducing the incidence of this type of meningitis by greater than 99%. Multiple combinations of the Hib vaccine with other vaccines (DTaP/Tdap, polio, and hepatitis B) are available in the United States, and the WHO has approval a pentavalent vaccine (Tdap-HepB-Hib) for use in developing countries.

A similar success story has recently occurred for invasive diseases caused by *Streptococcus pneumoniae,* which is a major cause of otitis media, bacteremia, and meningitis in infants, the elderly, and immunocompromised individuals. A heptavalent conjugate vaccine (called Prevnar) based on different capsule polysaccharides has reduced the frequency of pneumococcal invasive diseases in developed countries, and there are attempts to make this vaccine available in developing countries. In addition, a 13-valent pneumococcal vaccine (called Prevnar 13) is now available in the United States that protects against additional common serotype strains of *S. pneumoniae.* For adults at high risk for pneumococcal disease (i.e., those who are smokers or alcoholics, who have chronic lung, liver, or cardiovascular disease, or who are elderly), Prevnar 13 or a 23-valent vaccine (Pneumovax), which contains capsule polysaccharides from 23 serotypes of clinical isolates, can be administered.

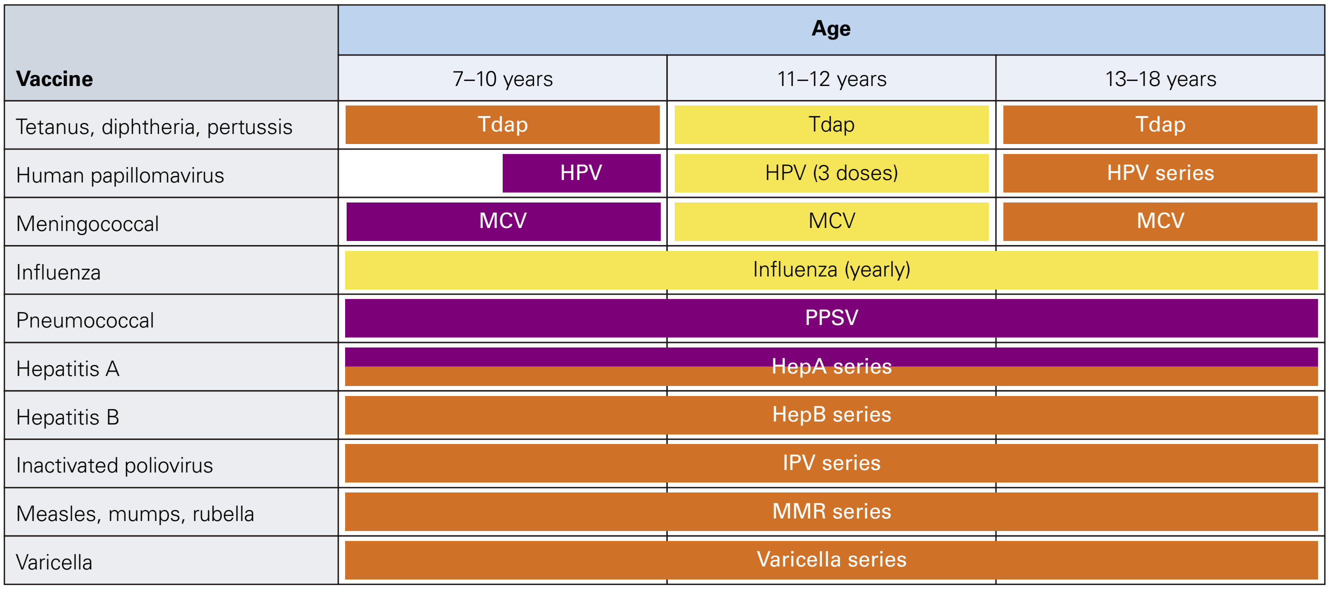
Recommended immunization schedule for persons aged 0 through 6 years —United States • 2016



<table>
<tr><th rowspan="2">Vaccine</th><th colspan="11">Age</th></tr>
<tr><th>Birth</th><th>1 month</th><th>2 months</th><th>4 months</th><th>6 months</th><th>12 months</th><th>15 months</th><th>18 months</th><th>19–23 months</th><th>2–3 years</th><th>4–6 years</th></tr>
<tr><td>Hepatitis B</td><td>HepB</td><td colspan="2">HepB</td><td></td><td colspan="4">HepB</td><td></td><td></td><td></td></tr>
<tr><td>Rotavirus</td><td></td><td></td><td>RV</td><td>RV</td><td>RV</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Diphtheria, tetanus, pertussis</td><td></td><td></td><td>DTaP</td><td>DTaP</td><td>DTaP</td><td></td><td colspan="2">DTaP</td><td></td><td></td><td>DTaP</td></tr>
<tr><td><i>Haemophilus influenzae</i> type b</td><td></td><td></td><td>Hib</td><td>Hib</td><td>Hib</td><td colspan="2">Hib</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Pneumococcal</td><td></td><td></td><td>PCV</td><td>PCV</td><td>PCV</td><td colspan="2">PCV</td><td></td><td></td><td></td><td></td></tr>
<tr><td>Inactivated poliovirus</td><td></td><td></td><td>IPV</td><td>IPV</td><td colspan="4">IPV</td><td></td><td></td><td>IPV</td></tr>
<tr><td>Influenza</td><td></td><td></td><td></td><td></td><td colspan="7">Influenza (yearly)</td></tr>
<tr><td>Measles, mumps, rubella</td><td></td><td></td><td></td><td></td><td></td><td colspan="2">MMR</td><td></td><td></td><td></td><td>MMR</td></tr>
<tr><td>Varicella</td><td></td><td></td><td></td><td></td><td></td><td colspan="2">Varicella</td><td></td><td></td><td></td><td>Varicella</td></tr>
<tr><td>Hepatitis A</td><td></td><td></td><td></td><td></td><td></td><td colspan="4">HepA (2 doses)</td><td></td><td></td></tr>
<tr><td>Meningococcal</td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

🟨 Range of recommended ages for all children except certain high-risk groups

Recommended immunization schedule for persons aged 7 through 18 years —United States • 2010



<table>
<tr><th rowspan="2">Vaccine</th><th colspan="3">Age</th></tr>
<tr><th>7–10 years</th><th>11–12 years</th><th>13–18 years</th></tr>
<tr><td>Tetanus, diphtheria, pertussis</td><td>Tdap</td><td>Tdap</td><td>Tdap</td></tr>
<tr><td>Human papillomavirus</td><td>HPV</td><td>HPV (3 doses)</td><td>HPV series</td></tr>
<tr><td>Meningococcal</td><td>MCV</td><td>MCV</td><td>MCV</td></tr>
<tr><td>Influenza</td><td colspan="3">Influenza (yearly)</td></tr>
<tr><td>Pneumococcal</td><td colspan="3">PPSV</td></tr>
<tr><td>Hepatitis A</td><td colspan="3">HepA series</td></tr>
<tr><td>Hepatitis B</td><td colspan="3">HepB series</td></tr>
<tr><td>Inactivated poliovirus</td><td colspan="3">IPV series</td></tr>
<tr><td>Measles, mumps, rubella</td><td colspan="3">MMR series</td></tr>
<tr><td>Varicella</td><td colspan="3">Varicella series</td></tr>
</table>

🟨 Range of recommended ages for all children except certain high-risk groups

🟧 Range of recommended ages for catch-up immunization

🟪 Range of recommended ages for certain high-risk groups

Figure 17-7. Recommended immunizations. The bars represent the optimal time range for administering vaccines and illustrate the complexity of the overall vaccination program. The recommendations, which are available from the website of the Centers for Disease Control and Prevention, are adjusted each year to reflect new or improved vaccines. **(Top panel)** Timing of recommended childhood vaccinations. **(Bottom panel)** Recommended vaccinations for ages 7 through 18 years based on health conditions.

Box 17-2.

Having an Effective Vaccine Is Not Enough: Missed Opportunities for Vaccination

In developed countries, the delivery of vaccines to the general public has been relatively effective. In the early 1990s, U.S. Department of Health and Human Services was not satisfied with the level of coverage. They established an official *Healthy People 2010* project goal for the year 2010, where at least 90% of all young children should have been vaccinated with DTaP, MMR, polio vaccine, and Hib, and at least 70% vaccinated with HBV. A study of vaccination levels in the United States from 2008 (Data from CDC. 2009. *MMWR* **58**:921–926) showed that, among families surveyed, approximately 76% of children had received the recommended number of DTaP, polio, Hib, MMR, and HBV vaccine administrations by the recommended age. While many states exceeded the goals, there was considerable state-to-state variation (from 82% to 59%), which was deemed worrisome. This study of vaccine usage revealed that the percent coverage was even worse among low-income urban populations than among other populations. The study also revealed that many of the children who had not been vaccinated could have been vaccinated in a timely manner, because they had passed through the health care system as infants and young children.

Why were these opportunities missed? For one thing, not all health care providers were routinely checking vaccination status of all young patients during each visit. This was especially true in cases where the child was brought in for treatment of an illness rather than for a routine checkup. For another, many health care providers seemed to think that illness precluded vaccination. There are some contraindications, but most childhood illnesses, particularly mild ones, do not preclude vaccination. Finally, advantage was not being taken of the fact that the vaccines listed in Table 17-3 can be administered simultaneously. Several recommendations were made to reduce these missed opportunities and help reach the 2010 goal:

(1) Accurate vaccination records should be maintained and should be easily accessible to health care providers.

(2) Vaccination status should be checked for every infant and young child during every visit to a health care provider.

(3) Health care providers should be made aware that mild illness does not preclude vaccination.

(4) Needed vaccines should be administered simultaneously.

Once the problem of under-vaccination was recognized, the CDC launched an advertising campaign to alert parents and physicians to the problem. Despite this campaign, which definitely helped raise public awareness, reports continue to come in of under-vaccination of certain populations. Further investigations of the reason for the continued under-vaccination rate in certain settings revealed that since physicians were not reimbursed adequately for vaccinations, even with the vaccine being provided free, they were routinely referring parents to vaccination clinics, thus necessitating another clinic visit and another wait in line. Even parents who are devoted to their children have limits—especially when they see the pediatrician acting as if vaccination were low on the scale of things to worry about.

Since infants and young children are the ones at highest risk for the severe form of many of these diseases, which occur before school begins, one strategy implemented to increase the percent of children vaccinated was to require that the vaccinations be completed before a child enters elementary school. This strategy has actually been quite effective because the median percentage of children entering school who have received the full vaccine series is now over 95% for all five vaccines in all of the states.

Source:

CDC. 2016. Vaccination coverage among children in kindergarten—United States, 2015–2016 school year. *MMWR* **65:**819–821.

Vaccine "Less-than-Success" Stories

The list of successful vaccines is rather pathetic if viewed from a historical perspective. DTP/DTaP has been around for years, as has the polio vaccine. With the exception of a few vaccines, such as the Hib, HepB, flu, human papilloma virus (HPV), pneumococcal, and meningococcal vaccines, the number of novel vaccines have been few and far between. Despite considerable funding for infectious disease research and technological breakthroughs that should have accelerated vaccine development, efforts of scientists have still not yielded suitably effective vaccines to prevent a number of globally threatening diseases, including tuberculosis, HIV, malaria, hepatitis C virus (HCV), *Neisseria meningitidis* serotype B, Group A and B *Streptococcus*, dengue, and other viral and bacterial infections. These diseases have been extremely challenging to control using traditional vaccine approaches. Clearly, not all vaccines created are successful and many have failed. Vaccine failure is when someone develops the disease despite being vaccinated against that disease.

Vaccines fail primarily for three reasons. One is failure to elicit the anticipated protective immune response. Three of the great vaccine disappointments of the late 20th century have been the failure, despite considerable funding support and herculean efforts, to develop effective vaccines against salmonellosis, cholera, and tuberculosis. Despite major advances in our understanding of how the bacteria that cause these diseases interact with the human body, there must still be important missing pieces in the picture, because numerous candidate vaccines have failed to show efficacy in clinical trials. So-far-unsuccessful attempts to develop vaccines effective against HIV infection and malaria can be added to this list. Here too, it is frustrating and perplexing to note that expenditure of huge amounts of time, money, and effort has not produced a better outcome. Clearly, scientists still have a lot more to learn about the host-pathogen interaction to ensure success in developing successful prevention strategies for many diseases.

In some cases, the lack of efficacy is understandable. For example, the *Neisseria* bacterium that causes gonorrhea is constantly changing the amino acid composition of its surface proteins, the ones targeted by opsonizing antibodies (see chapter 7). This type of rapid antigenic variability makes it very difficult to elicit the type of immunological response that will clear the bacteria from an infected person and protect from subsequent exposure. Similarly, HIV not only rapidly changes the surface proteins that it uses to enter cells from the outside, but also targets immune cells that are supposed to fight infection. The HIV genome integrates into the chromosomes of these cells, where it remains latent for many years. Other intracellular bacteria can move from cell to cell and avoid antibodies elicited by a vaccine designed to prevent bacterial attachment to cell surface receptors.

A second reason for vaccine failure is adverse side effects. Although vaccines used in national immunization programs are considered safe and effective, vaccines are not risk-free, and complications will occasionally occur following vaccination. Public trust in vaccine safety is key to the success of vaccination programs. An adverse event following immunization is any untoward medical occurrence after immunization; however, adverse events do not necessarily have a causal relationship with the use of the vaccine. One type of adverse side effect that is sometimes encountered with vaccines is allergic reaction to components of the vaccines. Estimates of allergic reactions range from 1 in 50,000 for immediate IgE-mediated hypersensitivity to 1 in 1,000,000 for delayed T cell-mediated hypersensitivity, autoimmunity, or even life-threatening anaphylaxis. Although these events are relatively rare and may or may not be directly related to vaccine administration, a person's perceived connection may negatively impact that person's willingness to undergo additional vaccinations.

An adverse side effect was the downfall of a vaccine against a common type of infant diarrhea caused by rotavirus. The vaccine was very effective in preventing infant diarrhea caused by rotaviruses, but a few infants developed an intestinal blockage as a side effect, which caused the vaccine to be abruptly withdrawn from use during clinical trials. In developed countries, rotavirus diarrhea can be treated effectively by replacing fluids lost during the diarrheal phase. Thus, in this case, the protection afforded by the vaccine was deemed insufficient to justify the risk. On the other hand, in developing countries where the availability of emergency medical rehydration treatment is much more limited, parents may find the vaccine more appealing, because the infant death rate due to diarrheal disease is so much higher.

Unarguably the worst nightmare for those who strive to develop a vaccine is when the vaccine fails due to its administration unexpectedly making the disease worse. The classic example of this type of vaccine failure is the human respiratory syncytial virus (HRSV) vaccine. RSV attacks infants in their first months of life and is a major cause of hospital admissions of infants under the age of 3 months. A vaccine that showed promising results in animals was administered to infants in the early stages of clinical trials conducted in the late 1960s. These trials were abandoned abruptly when it became apparent that vaccinated infants were getting a more severe form of respiratory disease than unvaccinated infants. Why this happened is still not entirely clear, but this example is another indication of how little scientists understand the immune response, despite continuing breakthroughs in this area. Nevertheless, several newer vaccines (subunit, live-attenuated, and vector-based vaccines with alternative vaccination routes) are currently being explored. More recently, a very promising vaccine against strains of *Chlamydia trachomatis*, an intracellular bacterial pathogen that causes sexually transmitted genital infections in the developed world and blindness (trachoma) in certain areas of the developing world, made the eye infections in vaccinated children worse than those in unvaccinated children. More recent anti-*Chlamydia* vaccine efforts have focused on the use of live, attenuated bacterial strains that use primate models of infection for evaluation of ocular pathology.

Vaccines can also fail when multiple vaccinations do not produce an adequate immune response. One cause of this could be inactivation of the vaccine during formulation by the manufacturer or through poor handling prior to administration. Another cause is interference with vaccine antigen presentation due to the presence of maternal antibodies that normally protect neonates. Some pathogens do not possess dominant epitopes that are easily neutralized or used for opsonization by antibodies that are elicited from a vaccine. Other pathogens produce virulence factors that actively dampen or misdirect the immune response (see chapters 11 and 12), such that even with a strong immune response from a vaccine, the pathogen is not effectively controlled.

Just as the earlier discoveries that vaccination could protect people from such a devastating infectious disease as smallpox revolutionized preventive medicine, recent discoveries that explain why some vaccines cause side effects, do not protect, or even make the symptoms of a disease worse are beginning to revolutionize vaccine development. In addition, the enormous insights about how the immune system works that have been achieved in the past decade are enabling vaccinologists to provide safer and more efficacious strategies for new vaccines in the future.

A New Age of Vaccine Development: Making Vaccines Better

Identifying an immunogen is just the first step that may lead to actually generating an effective vaccine. As mentioned previously, to move to the next step in vaccine development, an effective vaccine formulation must be safe with minimal side effects; it must also be able to elicit a strong, long-lasting, and appropriate immune response that is protective against future exposure to the native pathogen. It is, however, important to point out that vaccination does not guarantee complete protection from a disease. Sometimes the host immune system does not respond adequately or at all due to a number of host factors, such as: a lowered immune competence due to age, pregnancy, or other underlying diseases (e.g., diabetes, steroid use, HIV, cancer, or use of immunosuppressants); absence of B cells capable of generating antibodies to that particular antigen; or absence of strong T cell or B cell epitopes on the antigen. So, bearing these factors in mind, we will now consider ways in which vaccinologists are attempting to improve existing vaccines and develop new vaccines.

Approaches to Enhancing Immunogenicity

Eliciting the correct type of protective immune response is essential for the success of any vaccine. Vaccines against extracellular versus intracellular bacterial pathogens need to elicit different types of immunity, including appropriate Th1, Th2, and/or Th17 responses to direct the correct $CD4^+$-mediated antibody responses and/or $CD8^+$-mediated CTL responses. In the case of many extracellular pathogens, especially those that produce protein toxins or have conserved cell-surface protein antigens, protective immunity can often be achieved through vaccines that result in stimulation of a strong humoral response and, as noted previously, these types of vaccines have been the most successful to date. Vaccines against intracellular pathogens, where cellular immunity is critical for protection, have been much more challenging to develop. Unlike extracellular pathogens, intracellular pathogens in general have multiple virulence factors that contribute to pathogenesis, and, because they are intracellular, these potential antigens are not always easy to identify and often are not available *in vivo* to the antibodies that might be generated against them. There are a few exceptions, such as the case of *Listeria monocytogenes*, where a $CD8^+$ CTL response to a single protein antigen (listeriolysin O) is sufficient to provide protective immunity. But, as a rule, it is necessary to immunize with a mixture of antigens to elicit a protective response against most intracellular pathogens.

Adjuvants

One strategy to improve the immunogenicity of vaccines is the addition of **adjuvants**, which are components added to the vaccine formulation that serve as both **antigen delivery systems** and **immune potentiators** to induce stronger immune responses than the antigen alone. Adjuvants can also be used to help direct the immune system toward a desired Th1, Th2, and/or Th17 response. Table 17-4 summarizes the various roles that adjuvants play in vaccines.

Importance of Adjuvants. Historically, live attenuated and whole-cell vaccines did not generally require the use of synthetic adjuvants because the crude cellular contents of the killed whole organism or the live attenuated organism, or the viable attenuated virus served that function adequately. Nowadays, however, the components of acellular or subunit vaccines tend not to provoke strong immune responses; thus, they require the addition of adjuvants to improve delivery and immunogenicity as well as to achieve other more specific effects that might direct the appropriate immune response. From the foregoing description, it might appear that subunit vaccines like DTaP consist solely of the purified protein antigens. Although proteins given alone can produce an immune response, they work much better if they are given in conjunction with adjuvants to stimulate, modify, or augment the immune response to the vaccine.

In the case of peptide vaccines, adjuvants are also essential to elicit a strong immune response because peptides given alone not only do not elicit much of an immune response, but also can cause the body to ignore the epitope through a process known as **induced tolerance** (or **anergy**). This anergy is a consequence of antigen-induced cell death of activated, antigen-specific T cells. Adjuvants, through an unknown mechanism, can increase the half-life of activated T cells and counter this effect.

Freund's Adjuvant. First developed in the 1940s, **Freund's complete adjuvant** is composed of inactivated and dried *Mycobacterium tuberculosis*, the causative agent of tuberculosis emulsified in mineral oil or paraffin. It is highly effective in stimulating cell-mediated immunity. But, because it stimulates production of the cytokine tumor necrosis factor (TNF), its use, even in animals, is now restricted due to the associated pain and tissue damage. **Freund's incomplete adjuvant** is the same adjuvant, but without the mycobacterial components, and is still used in many veterinary vaccines.

Aluminum Salts. Although in other countries (and now more and more in the United States) a number of vaccine adjuvants have been approved for human use (see the fol-

Table 17-4. The roles of adjuvants in vaccines

1. Enhanced humoral immunity
a. increased antibody production and sustained antibody titer
b. enhanced bactericidal, viral, and toxin neutralization or inactivating properties
c. induction of Th2 cytokines and B-cell memory
2. Enhanced cell-mediated immunity
a. induction of Th1 cytokines (e.g., IFN-γ)
b. induction of T-cell memory
3. Enhanced mucosal immunity
a. increased stability for oral administration
b. enhanced mucosal uptake
c. enhanced stimulation of innate immunity
d. enhanced stimulation of IgA/sIgA production
4. Reduced reactogenicity
5. Antigen components
a. provide broad protection (cross-reactivity)
b. enable multi-antigen combinations in one administration
c. reduced amount of antigen dose in vaccine
d. reduced number of doses necessary for long-lasting immunity
e. minimize competition between antigens in combination vaccines that reduces efficacy
6. Enhance immune responses in young children, the elderly, or immunocompromised individuals, who have poor immunity

lowing), until 2009, **alum** was the only approved adjuvant licensed for human use in the United States. Alum is composed of particulate aluminum salts, such as $Al(OH)_3$ or $AlPO_4$. For a long time, it was thought that alum, which has a very long half-life at the site of injection, enhanced the immune response of antigens by allowing for gradual release of the adsorbed antigen and thereby increasing exposure of antigen-presenting cells (APCs) to the antigen. This view has now been challenged by evidence that radiolabeled antigen is released very rapidly from the alum complex and disseminates away from the site of injection.

Recent evidence suggests that alum acts by promoting antigen uptake into dendritic cells (DCs) or other APCs and by activating innate immune reactions and cytokine release that stimulate humoral activity via Th2 responses (IgG1, IgE, IL-4, and IL-5), but the exact mechanism of its action is still not clear. The primary advantage of using alum is its long history of safety for human use. Although alum is a good adjuvant for toxoid-based vaccines that rely on generation of Th2 humoral immunity, its major limitation is that it is a poor inducer of Th1 and cell-mediated immune responses. There is also a limit on the total amount of alum that can be administered per dose, which curtails its use for multicomponent vaccine formulations.

Vaccines containing alum, as well as those comprised of oil-in-water emulsions, cannot be sterilized by filtration, freezing, or lyophilization, and so have storage limitations as well. These adjuvant formulations need to be filter-sterilized by passing through a 200-nm-pore-sized filter to be suitable for vaccines that do not need refrigeration. This requirement is not easily met and requires considerable formula testing.

Triterpenoid-based Adjuvants. Plant-derived triterpene glycosides (**saponins**) have gained popularity as vaccine adjuvants because they can elicit strong, long-lasting Th1, Th2, and cell-mediated immunity, including CTL responses. The most widely used saponin-based adjuvants are **QuilA** and its derivative **QS-21** (Figure 17-8), which are mixtures of saponins that are prepared in oil-in-water emulsions. Unfortunately, the high toxicity and undesirable hemolytic side effects, as well as instability in water, have so far restricted their use to veterinary vaccines (more on this later).

Squalene, on the other hand, is a triterpenoid that is a precursor of cholesterol and other steroid hormones. Since it is a natural component found in humans and is also biodegradable, it has been used to develop the first new adjuvant to gain FDA approval for vaccine use in humans since alum. The adjuvants **MF59** and **AS03** are licensed for human use. MF59 is an oil-in-water emulsion of squalene and two non-ionic surfactants (Tween 80 and Span 85) as stabilizers, while AS03 is an emulsion of squalene with α-tocopherol (also known as vitamin E) (Figure 17-9). Both have been included in influenza vaccines available in many countries worldwide. Postmarketing surveillance data have confirmed that MF59 is safe and elicits higher antigen-specific antibody titers than alum and also promotes innate immune responses by recruiting and augmenting antigen uptake DCs, monocytes, and macrophages to stimulate CD4+ T cells and high antibody titers. MF59 has now been evaluated in clinical trials for a number of other antiviral vaccines and, so far, has shown no serious adverse effects and in general is well tolerated. AS03, on the other hand, has shown some adverse side effects that are under investigation.

Virosomes. Influenza virus-derived virosomes are another market-approved, efficient antigen carrier and adjuvant for vaccines. Virosomes are vesicles (nanoparticles) composed of a lipid membrane with membrane-bound viral proteins, mostly hemagglutinin and neuraminidase. Virosomes retain the host cell receptor-binding and pH-dependent membrane fusion activity of the virus, but lack the viral genetic material. These properties enable them to deliver their encapsulated antigens to DCs and other APCs. Because virosomes present antigens via both MHC-I and MHC-II, they confer both humoral antibody and cell-mediated immunostimulatory properties (Figure 17-10). Virosomes have the advantage of encapsulating and stabilizing the antigens, thereby protecting them from degradation. They also have excellent safety records and are suitable for infants, the elderly, and immunocompromised individuals. Another

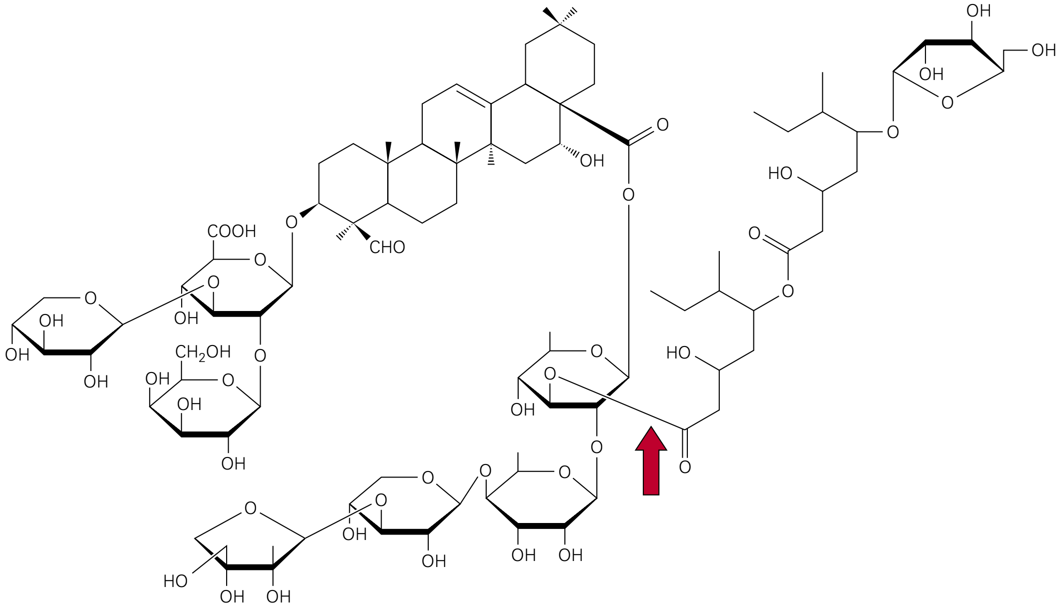


Figure 17-8. **Chemical structure of the saponin-based adjuvant QS-21.** The arrow points to the location of the acyl side chain group that is responsible for stimulation of the Th1 and CTL responses, and unfortunately also for its toxicity due to cytolytic activity.

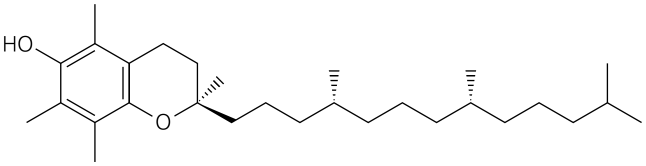


Figure 17-9. **Structure of α-tocopherol (vitamin E).**

advantageous property of virosomes is the possibility of incorporation with other lipophilic adjuvants to provide additional immune-enhancing functions, and studies are under way to explore this possibility.

Liposomes and Microspheres. Liposomes are a particularly interesting type of adjuvant that may solve the problem of having to administer DTaP so many times. **Liposomes** incorporate the antigens and adjuvant components (discussed more later) in synthetic lipid membranes and provide many different ways to provide the desired immune outcome (Figure 17-11). Another antigen delivery system used is encapsulation of the antigens in **microspheres** (or **microcapsules**), which are made of resorbable suture material or some other inert substance that gradually breaks down in the body. For example, microparticles of biodegradable polymers such as poly(lactide-co-glycolide) entrap the protein or polysaccharide antigen, and encapsulated antigens are then released slowly over a period of time to increase exposure time with the immune system. By administering a mixture of microspheres of differing sizes, it is hoped that the vaccine will be released over a long enough period so that a memory response will be elicited with only one or two injections.

Programming Adaptive Immunity

Another strategy to improve the immunogenicity of vaccines is to alter the mode of delivery of the vaccine so as to elicit the appropriate type of immune response at the site of infection. Although adjuvants have long been known to shape the immune response, it is only recently that the underlying mechanisms by which they do so have begun to be unraveled. Consequently, alternative strategies can now be conceived for manipulating this newfound information using the myriad of newly determined properties of various adjuvants, so as to elicit the desirable outcome of an immune response.

Targeting Dendritic Cells and Innate Immune Sensing. A key event that triggers the immune response is when the immune system "senses" the vaccine antigen or microbe. Activation of DCs and other APCs translates into release of cytokines and activation of antigen-specific T cells and B cells, which consequently modulate the strength, quality, and persistence of the adaptive immune response. Critical to this process is triggering the innate immune responses of DCs and APCs through Toll-like receptors (TLRs), as well as C-type lectins and NOD proteins. Triggering distinct TLRs activates DCs to elicit different cytokine profiles that differentiate Th1 versus Th2 responses (see Figure 3-5).

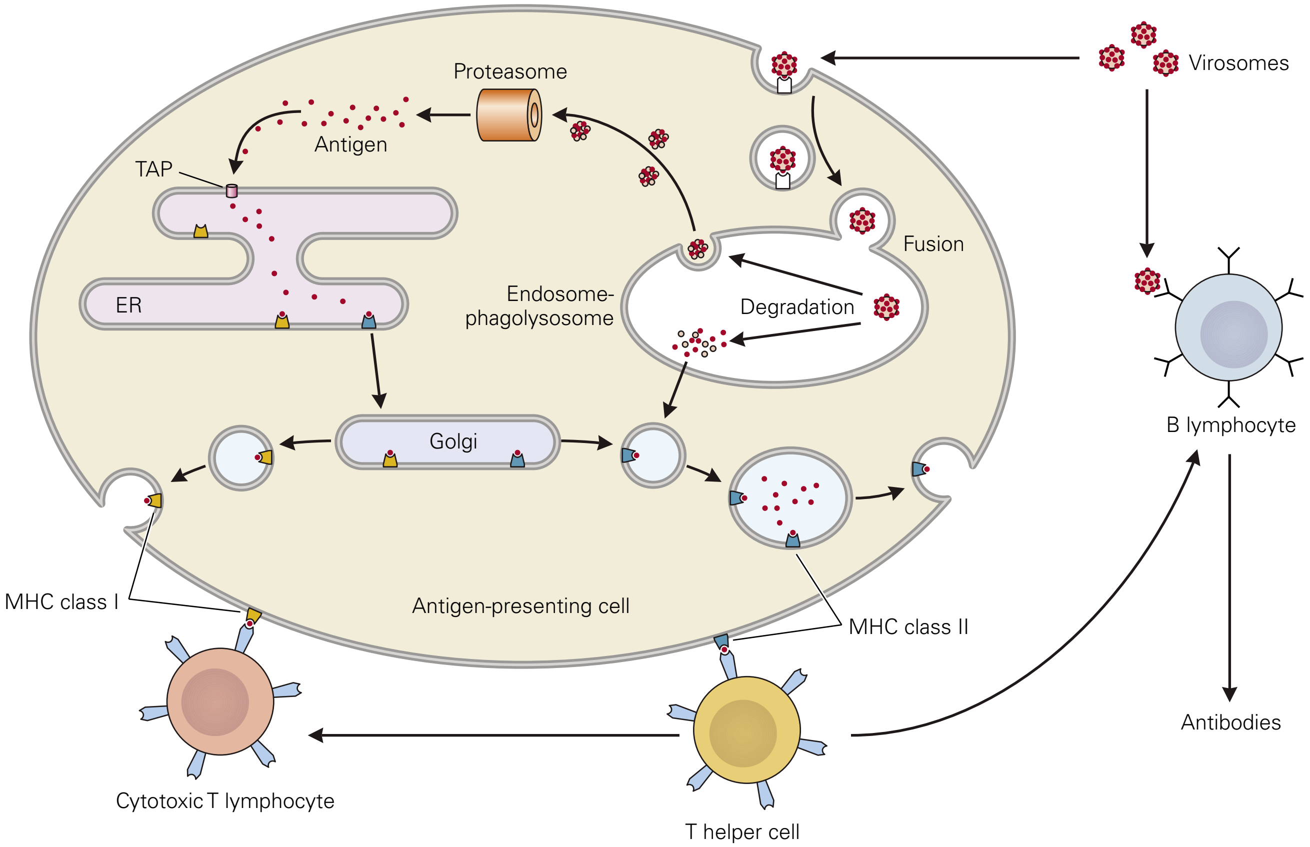


Figure 17-10. Virosome interactions with immune cells. Through multiple binding interactions of hemagglutinin on the virosome surface with APCs, the virosomes are taken up and the antigens trapped (or encapsulated) within the virosomes are processed through both MHC I and MHC II pathways, activating T helper cells and stimulating both Th1 and Th2 responses. Adapted from Wilschut J. 2009. *Immunol Lett* **122:**118–121, with permission from Elsevier.

Different TLR agonists are currently being tested as adjuvants to tailor the immune response so that optimal protection is induced. For example, the TLR4-specific agonist LPS (in the form of detoxified **monophosphoryl lipid A [MPL]** obtained from *Salmonella*) is being combined with other adjuvants, such as alum or the saponin-based QS-21, for vaccine formulations. Other TLR agonists, such as flagellin (linked to antigen), **poly(I:C)** dsRNA analogs, and unmethylated **CpG oligonucleotides** (a form of bacterial dsDNA that is underrepresented in humans), which stimulate TLR5, TLR3, and TLR9, respectively, are currently being tested in various clinical trials.

By combining new immunostimulatory adjuvants (QS-21, MPL, CpG) with classical adjuvants (alum, liposomes, oil-in-water emulsions), improved **Adjuvant Systems (AS)** can be formulated. When the saponin QS-21 was initially found to have too much toxicity, efforts were made to abrogate the intrinsic lytic activity by combining it with cholesterol-based liposomes. Although the fear was that its strong immunostimulatory properties were linked to the lytic activity, this was not found to be the case, and indeed the new formulation proved not only nontoxic but also immunostimulatory (Figure 17-12). **AS01** is a combination of QS-21 with the adjuvants MPL and liposomes, while **AS02** is the same as **AS03** plus MPL and QS-21. AS01 and AS02 increase antibody titer and stimulate Th1 responses. Another such adjuvant, **AS04**, which consists of MPL absorbed on alum, has also been approved for human vaccines. AS04 has the added benefit of inducing a strong Th1 response by production of IL-2 and IFN-γ, which cannot be stimulated by alum alone.

Heat shock proteins and antigens with **RGD tags** (Arg-Gly-Asp peptide motifs that act as ligands for cell surface **integrins**) specifically bind to and activate DCs, and so are being explored as potential adjuvant components. Activation of DCs increases their ability to process and present antigen and to attract and activate T cells through cytokine secretion. Because of this, recent vaccine and adjuvant improvement strategies include using **cytokine technology** to modulate immune responses. For example, IL-12 has strong adjuvant activity when administered with antigen because it stimulates a Th1 specific response. In chronic infections, such as those caused by the intracellular parasite *Leishmania*, the severity of the disease is determined by whether

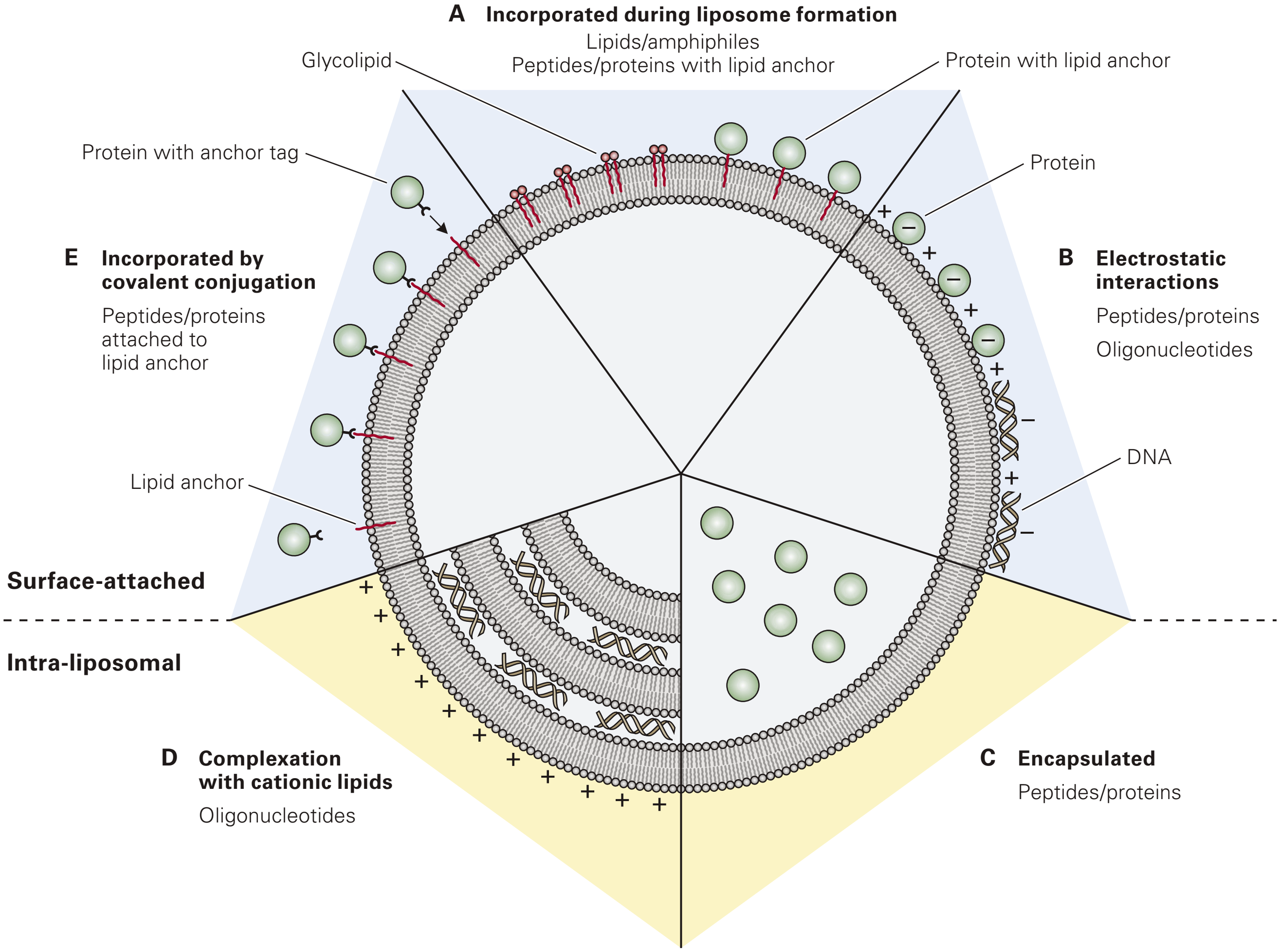

A Incorporated during liposome formation
Lipids/amphiphiles
Peptides/proteins with lipid anchor
Glycolipid
Protein with lipid anchor
Protein with anchor tag
Protein
E Incorporated by covalent conjugation
Peptides/proteins attached to lipid anchor
B Electrostatic interactions
Peptides/proteins
Oligonucleotides
DNA
Lipid anchor
Surface-attached
Intra-liposomal
D Complexation with cationic lipids
Oligonucleotides
C Encapsulated
Peptides/proteins

Figure 17-11. Different strategies for incorporation of antigens and adjuvants in liposomes. Different formulations and structures of liposomes can be employed to tailor the desired antigen delivery and adjuvant immunomodulating properties of the vaccine. **(A)** Hydrophobic molecules and lipids can be incorporated into the lipid bilayer. **(B)** Peptides, proteins, and nucleotides can be electrostatically adsorbed to the surface of oppositely charged liposome surfaces. **(C)** Peptides and proteins can be trapped (or encapsulated) within the lumen of liposomes. **(D)** Nucleotides can be complexed with cationic lipids into multiple lipid bilayers. **(E)** Peptides and proteins can be attached to the liposome surfaces by covalent conjugation to functionalized lipid anchors. Adapted from Tandrup Schmidt S, Foged C, Korsholm KS, Rades T, Christensen D. 2016. *Pharmaceutics* **8:**e7, with permission.

Th1 or Th2 mediates the immune response. The ability to manipulate the Th1 versus Th2 responses could enhance protection and reduce pathology of the disease.

Epitope-based Synthetic Vaccine Strategies. Recombinant DNA technology revolutionized the development of vaccines by allowing for the production and testing of recombinant nontoxic antigens, such as site-directed, catalytically inactive toxins instead of toxoids (chemically treated or heat-inactivated toxins). The tremendous leap in understanding of the mechanisms underlying protective immunity that we have achieved in recent years has led to the development of a new class of recombinant vaccines. This newfound knowledge coupled with the power of molecular biological techniques enabled protein engineers to change the conformation and structure of the antigen by genetic manipulation to enhance DC-binding properties to stimulate desired immune responses and to enhance antigen presentation through the design of multiple **epitope-based targeting signals** into the peptide sequence.

Examples of such entirely synthetic recombinant vaccines are shown in Figure 17-13. As mentioned previously, the addition of the RGD peptide (an integrin-binding motif) to the recombinant vaccine protein allowed for targeting of the antigen to DCs. Inclusion of known MHC I-restricted epitopes that activate the desired T helper cells in the recombinant vaccine protein allowed for modulation of the appropriate cellular immune response. Attaching a TLR-specific

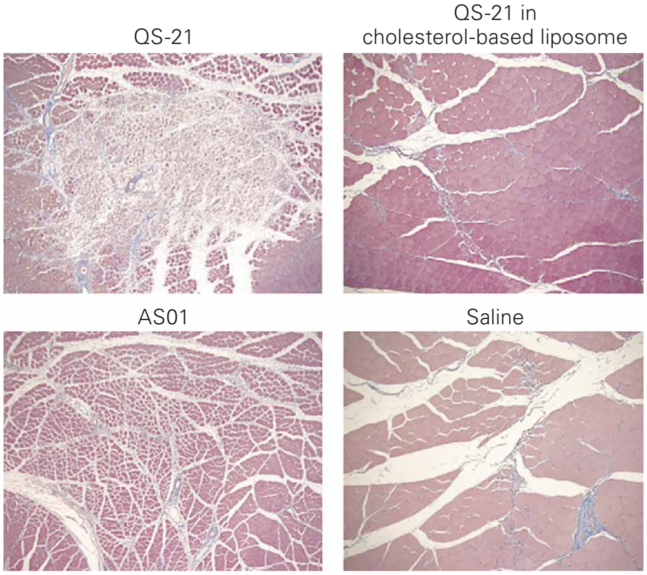


Figure 17-12. Combination adjuvant formulations for improved vaccines. The toxic cytolytic activity of the saponin QS-21 on tissues (top left panel) was abrogated when combined in cholesterol-based liposomes (top right panel). This formulation was then combined with the immunostimulant 3-deacylated monophosphoryl lipid (MPL) to become the AS01 adjuvant formulation (bottom left panel). Adapted from Garcon N, Di Pasquale A. 2017. *Hum Vaccin Immunother* **13**:19–33, with permission.

ligand to the antigen allowed for stimulation of appropriate innate immune responses. Incorporation of lysosomal protease cleavage sites in between known epitopes in the protein antigen allowed for enhanced antigen processing, especially through the MHC II pathway. These epitope-based strategies can also be combined with other vaccine delivery systems and adjuvants for greater flexibility in vaccine design.

Based on these concepts, it has been possible to design a number of entirely synthetic vaccines that are currently under clinical investigation. But, keep in mind that the multiple chemical steps needed to manufacture synthetic vaccines can add to their cost, which can impact their attractiveness to pharmaceutical companies.

DNA Vaccine Technology. In the foregoing text, all of the vaccines have been bacteria, viruses, proteins, polysaccharides, sugar–protein, or peptide–lipid conjugate antigens. A completely different approach to vaccination that has received a lot of attention is the injection of DNA vector containing a gene that encodes a vaccine protein antigen into human muscle cells, where it is expressed under a mammalian promoter. These vaccines are called **DNA vaccines**.

The DNA is either coated with or trapped (also called "encapsulated") within liposomes or microspheres, as described previously, for delivery via injection or, alternatively, the DNA is first adsorbed to gold particles and injected with an air gun into muscle tissue. Incredibly enough, foreign antigens are produced and displayed on the surface of the muscle cells. Although display of the foreign antigen lasts only a month or two, this is long enough to evoke a robust response. Because the display is temporary and localized, side effects associated with an autoimmune attack on self-cells are minimized. Also, the vaccine DNA is introduced into differentiated cells, not in the germ line, so it will not be passed on to subsequent progeny.

There are, however, some limitations with the use of DNA plasmids for vaccines and gene therapy. These limitations largely arise directly from how they are produced. In order to generate large quantities of DNA, plasmids are typically propagated in and harvested from special bacterial cloning strains. In order to grow in these bacteria, plasmids encode additional DNA elements like origins of replication (*ori*) and genes for selection, such as antibiotic resistance or auxotrophy. The presence of an *ori* might lead to inadvertent transfer of the DNA to other bacteria encountered in the host. On the other hand, once the plasmids enter host cells, they are nonreplicating episomes, which are diluted by cell division. This dilution means that expression of the encoded gene is only transient, potentially limiting the vaccine's effectiveness. Foreign bacterial DNA sequences can also lead to unexpected gene silencing, and the unmethylated CpG sequences found in bacterial DNA also have the potential to overstimulate TLR9-mediated inflammatory immune responses. Similarly, residual LPS (endotoxin) from the bacteria can overstimulate TLR4-mediated inflammation. Finally, because of the antibiotics used in propagating the DNA plasmids, the resulting DNA may contain residual antibiotics, which poses the risk of adverse reactions in individuals with allergies to the antibiotics.

The use of an air gun to deliver the DNA-coated gold particles has a number of important advantages. First, unlike live vaccines or subunit vaccines with most adjuvants, DNA on gold particles can be stored dry and need not be refrigerated. Second, pure plasmid DNA is much less expensive to prepare, formulate, ship, and store than pure protein antigens or viral particles. Third, DNA-coated gold particle-based vaccines do not require adjuvants or potentially viable viral particles as in the case of viral vaccines. So far, the results from this approach have caused considerable excitement and human trials are already underway.

Just as DNA vaccines appeared to be ready to revolutionize vaccination procedures, some troubling concerns have emerged centered around the fact that DNA is immunostimulatory. Scientists working on the disease lupus have long suspected that this autoimmune disease was caused by the body's immune reaction to its own DNA. Articles have begun to appear that question the safety of injecting DNA into human tissue. Basically, the claim is that certain DNA sequence motifs, which are found in bacterial DNA, elicit an

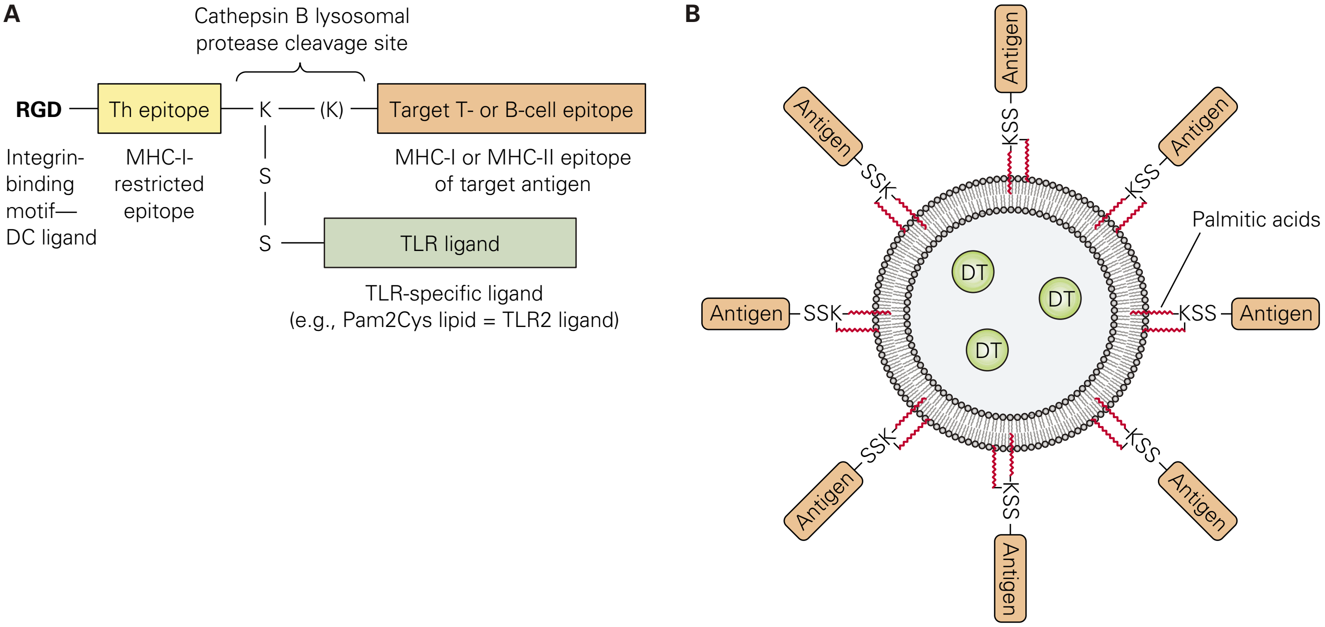

A
Cathepsin B lysosomal protease cleavage site
RGD
Th epitope
K
(K)
Target T- or B-cell epitope
Integrin-binding motif—DC ligand
MHC-I-restricted epitope
S
S
MHC-I or MHC-II epitope of target antigen
TLR ligand
TLR-specific ligand (e.g., Pam2Cys lipid = TLR2 ligand)
B
Antigen
KSS
SSK
DT
Palmitic acids

Figure 17-13. Ideal entirely synthetic epitope-based vaccines. Shown are potential ideal candidates for entirely synthetic recombinant vaccines targeting specific immune responses. **(A)** An entirely synthetic epitope-based vaccine could contain an RGD motif, which would target the antigen for DC binding and uptake. It would consist of at least two epitopes, one strong known Th-specific epitope at the N terminus and one or more B or T cell epitopes from the target antigen, which would elicit the desired CD4$^+$ or CD8$^+$ response, respectively. A lysosomal protease cleavage site (e.g., containing lysines for cathepsin B cleavage), which allows for optimal antigen processing, separates the epitopes. A TLR-specific ligand is also included by linking the ligand (such as a lipid adjuvant) via two serine residues to the ε-amino group of one of the lysine residues of the peptide containing the epitope. **(B)** Another approach is to combine epitope-based recombinant strategies with antigen-encapsulated liposomes. In this case, diphtheria toxoid (DT) is trapped (encapsulated) within liposomes to stimulate T cells, while the lipidated peptides, containing epitopes of targeted B cells attached to the N-terminus of the spacer KSS (Lys-Ser-Ser), are covalently coupled to two palmitic acid lipid molecules that allow their insertion into the liposome membrane.

immune response that might lead to an autoimmune response in some people, which could in turn lead to an increase in such diseases as lupus. It may prove to be the case that if DNA is injected into muscle tissue, not enough of it will get into the bloodstream to trigger an immune response. But, if these concerns prove to be correct, then it could adversely impact the application of this approach for gene therapy and vaccines.

A new, very promising strategy that alleviates some of these disadvantages of plasmids as DNA vaccines is the recent development of minimized DNA vectors or minivectors (Figure 17-14). Cloning the target gene sequences between *attB* and *attP* attachment sites located in the starting plasmid generates the DNA minivectors. Intramolecular recombination of the *attB* and *attP* sites by λ integrase produces two catenated vectors, which are then unlinked by topoisomerase IV to generate the DNA minivector that now harbors only the encoded target antigen gene. Unwanted bacterial sequences, such as *ori* or antibiotic selection genes, remain with the discarded larger plasmid vector. DNA minivectors are easier to obtain in highly purified form, have higher expression efficiencies, and have improved safety profiles. These DNA minivector-based vaccines are now being tested in preclinical trials as replacements for live attenuated viral-based vaccines.

Targeting Mucosal Immunity

Vaccine Vector Technology. One strategy for eliciting the correct immune responses against bacterial pathogens is to use molecular techniques to make a live, avirulent or attenuated bacterial vaccine strain that produces the protein antigen (or portions of it) on its surface. For example, since many pathogens gain entry into the body through mucosal surfaces, targeting mucosal immunity is a desirable approach for vaccine development against such pathogens. Vaccines based on killed whole cells or on isolated protein or polysaccharide antigens do not generally induce strong CD8$^+$ T cell responses, so live attenuated mutants are also the most effective strategy against pathogens that require cellular immunity for protection.

Avirulent bacterial vectors, such as attenuated, antigen-expressing *Salmonella* strains, are being tested as vaccines targeting mucosal immunity against gut pathogens. These vector cells bind to and invade M cells in the gut, where they

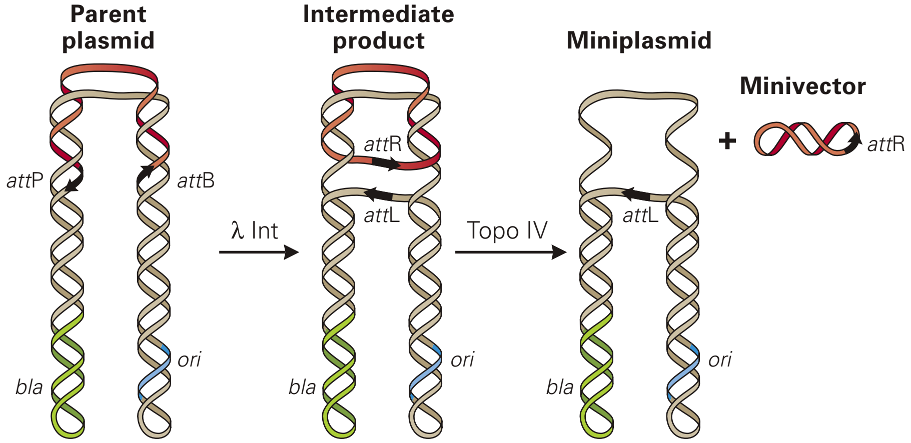


Figure 17-14. Generation of DNA minivectors. To generate the DNA minivectors, the target antigen sequence is cloned between the *attB* and *attP* attachment sites of the parent plasmid, containing the *ori* and the antibiotic selection gene for β-lactamase (*bla*). λ Integrase (λ Int) mediates intramolecular recombination of the *attB* and *attP* sites, producing two catenated vectors, which are resolved by topoisomerase IV (Topo IV) into a miniplasmid that retains the *ori* and *bla* gene and the minivectors that encode the target antigen gene. Adapted from Hardee CL, Arévalo-Soliz LM, Hornstein BD, Zechiedrich L. 2017. *Genes* **8**:E65, with permission.

interact with mucosal-associated lymphoid tissue (MALT) and stimulate sIgA as well as cellular immune responses. *Salmonella* has another advantageous feature that makes it a desirable candidate as a vaccine vector. It has type 3 secretion systems that could be used to deliver immunogenic antigens or epitopes directly into host immune cells. Other avirulent or attenuated strains of pathogens being explored as antigen-delivery vectors include *Vibrio cholerae*, *L. monocytogenes*, *Shigella* species, *Mycobacterium bovis*, and *Yersinia enterocolitica*. Bacteria found in the normal intestinal microbiota that are being explored include *Streptococcus gordonii*, *Lactobacillus* species, and *Lactococcus* species.

The anti-TB vaccine BCG (Bacillus Calmette-Guérin) is an attenuated strain of *M. bovis*, which causes a TB-like disease in humans and cattle that results in subsequent immunity to TB. Controversy over BCG's efficacy (ranging from 0% to 80% in field trials—now thought to be due to large gene deletions and other polymorphisms in some of the vaccine strain preparations that were used in the trials) and the problem that people vaccinated with it test positive in the TB skin test caused the disuse of BCG in the United States. However, because of the emergence of multidrug resistant TB since the 1990s, BCG is now administered to at-risk health care workers. BCG is also being investigated as a vaccine vehicle because of its extensive safety record and its ability to elicit a strong immune response. Efforts are underway to engineer BCG to display recombinant vaccine antigens against other pathogens on its surface.

A number of problems have been encountered with generation of effective live vaccines. One problem has been getting sufficient amounts of the recombinant foreign antigens or epitopes to be expressed on the bacterium's surface without overburdening the bacterium's metabolism and impeding its growth. Concerns about reversion also remain a primary obstacle to the use of live vaccines for many candidate targets, particularly for those that might come in contact with other related microbes where horizontal gene exchange could occur.

Another major difficulty is generating mutant strains of the bacteria that are avirulent or attenuated enough to be nontoxic and safe, yet still able to survive long enough in the body to generate a strong immune response. This requires a delicate balance that is extremely challenging to achieve. This has been greatly complicated recently with the realization that there is an abundance of bacteriophages in both the environment and the body and that these bacteriophages often carry virulence factors, which could result in converting the avirulent vaccine strain into a potentially deadly pathogen. One solution to this problem is the use of **psoralen** (Figure 17-15) plus UV treatment, which cross-links DNA. This blocks DNA synthesis, replication, and repair in the bacteria, but not protein synthesis or metabolism. These cells, which are killed but metabolically active, do not proliferate, but they remain alive and can still produce

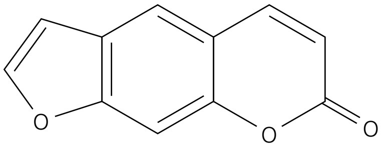


Figure 17-15. Chemical structure of psoralen, a UV-sensitive photoactivatable DNA cross-linker.

antigens and elicit strong immune responses. These vaccines not only deliver the targeted antigen epitopes to elicit the desired immune response, but also present all of the antigens of the pathogen to the immune cells, an advantage for cases where all of the features needed for protection are still not fully understood. On the other hand, vaccines based on whole cells have the complication of releasing LPS and other bacterial surface molecules that can overstimulate the innate immune response.

Toxins to Stimulate Mucosal Immunity. Another powerful adjuvant comes from an unexpected source: the bacterium that causes cholera (*V. cholerae*). *V. cholerae* produces a toxic protein complex called **cholera toxin (CT)**, which causes a massive dehydrating watery diarrhea (see chapter 12). For reasons that are still not completely clear, CT is a very effective stimulator of the antibody-specific immune response. There is currently considerable interest in developing vaccines that do not require needle injections but can be administered intranasally or by skin patches (discussed further in the next section). CT is being applied as an adjuvant to stimulate immune responses to vaccines administered through the skin.

Researchers are beginning to explore the use of catalytically inactive or attenuated toxins to deliver epitopes in candidate vaccines that require stimulation of both humoral and cell-mediated responses. Diphtheria toxin (DT) was the prototype of such "tools," but researchers have now expanded the repertoire to include other toxins, including CT, clostridial neurotoxins, and anthrax toxin. Since the B subunit of CT binds to M cells of the gut, linking an antigen to the B subunit will target the antigen for direct delivery to MALT. In the case of anthrax toxin (Figure 17-16), the protective antigen (PA) is able to deliver a variety of antigens that are coupled to the PA-binding N-terminus of the edema factor (EF) or lethal factor (LF) catalytic subunit.

Injection-Free Vaccines. There are some new vaccine developments that stimulate mucosal immunity via MALT that are very exciting. One such development is rather pedestrian in the sense that it applies to vaccine administration, but it is an advance that will be hailed as revolutionary by physicians, parents, and children alike: the shot-free vaccination. These developments may signal the end of the dreaded syringe.

One such development is **intranasal inoculation** of the vaccine. Nasal inoculation activates the nasal-associated lymphoid tissue (NALT), the upper respiratory tract equivalent of the intestinal gastrointestinal-associated lymphoid tissue (GALT). Nasal vaccines against influenza virus have come into widespread use recently. One major advantage of targeting BALT over GALT is that nasal inoculation does not require protection of the antigen from degradation in the harsh environment of the stomach (see Table 17-5). And, as we learned in chapter 3, mucosal immunity generated at one mucosal site in the body can provide protective responses at other mucosal sites.

Table 17-5. Pros and cons of oral versus nasal vaccines

VACCINE PROPERTY	ORAL	NASAL
Delivery	Easy	Simple device
Patient compliance	High	Moderate
Antigen digestion/ degradation	Extensive	Limited
Antigen uptake	Poor	Good
Immunogenicity	Poor	Modest to good
Immunological tolerance	Possible	Possible
Antigen dose required	High	Medium
Adjuvant required	Powerful mucosal	Mucosal
Targeting required	Mandatory	Helpful
Safety	Safe	Safe

Nasal influenza vaccines contain live attenuated viruses, which cannot replicate in most areas of the body because it is too warm, but they can replicate in the nose, which is a few degrees cooler than the rest of the body. Production of virus for these vaccines is currently a bottleneck, as exemplified by delays in distribution of vaccine against H1N1 "swine" flu, because the viruses are currently propagated in chicken eggs. Improvement in the technology to produce large amounts of virus for live attenuated influenza vaccines, as well as to inactivate the virus for the more traditional "flu shot," will undoubtedly continue to improve to allow rapid deployment to prevent pandemics.

A second development is a vaccine **patch** that contains many tiny prongs. The patch is applied to the skin surface to stimulate SALT, but the prongs do not penetrate far enough to encounter the epidermal neurons so that application of the patch is painless. Coupled with the use of CT as an adjuvant (noted earlier), administration of a vaccine using the patch could stimulate antibody-specific immune responses to various antigens via SALT. This response is often accompanied by a general mucosal immune response, a very desirable feature. And since your skin cannot develop cholera diarrhea, CT as an adjuvant should be safe.

Edible Vaccines. Still another interesting approach is the development of **edible vaccines**. The ability to genetically engineer plants has made it possible to produce plants that synthesize vaccine proteins. The original, and somewhat naïve, idea was that vaccination could occur by the simple act of ingesting the plant. Currently, edible vaccines are be-

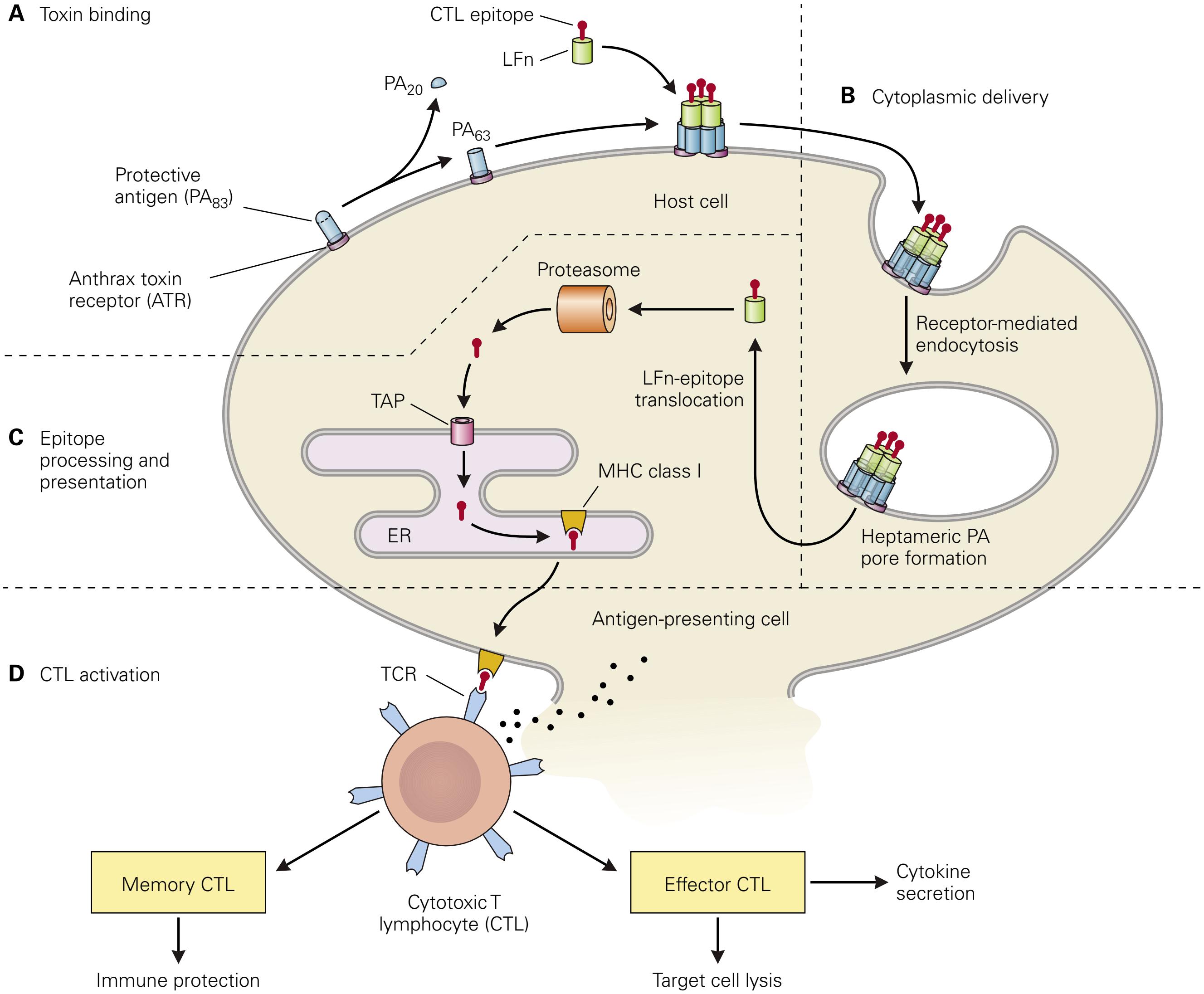

A Toxin binding
CTL epitope
LFn
PA$_{20}$
PA$_{63}$
Protective antigen (PA$_{83}$)
Anthrax toxin receptor (ATR)
Host cell
B Cytoplasmic delivery
Proteasome
Receptor-mediated endocytosis
LFn-epitope translocation
TAP
C Epitope processing and presentation
MHC class I
ER
Heptameric PA pore formation
Antigen-presenting cell
D CTL activation
TCR
Memory CTL
Effector CTL
Cytokine secretion
Cytotoxic T lymphocyte (CTL)
Immune protection
Target cell lysis

Figure 17-16. Delivery of vaccine antigens as fusions with bacterial toxins. Shown is an example of the use of anthrax toxin for vaccine delivery. Coupling a CTL-specific epitope to a catalytically inactive form of lethal factor (LF) of anthrax toxin allows for uptake, processing, and presentation of the epitope in antigen presenting cells. **(A)** Toxin binds to APC, **(B)** toxin is endocytosed and epitope is translocated into cytoplasm, **(C)** epitope is processed and presented on cell surface through MHC I complex, and **(D)** activation of CTL response. LFn, N-terminus of LF; PA, anthrax protective antigen; ER, endoplasmic reticulum; TAP, transporter associated with antigen processing; TCR, T cell receptor; CTL, cytotoxic T lymphocyte. Adapted from Shaw CA, Starnbach MN. 2003. *ASM News* **69**:384–389.

ing produced by a large number of plant species. Two major advantages of plant-based oral vaccines are the low cost of production and their ability to confer both mucosal (IgA) and systemic (IgG) immunity.

Several of these vaccines have been through small trials in humans to test safety. So far, they have been safe, but they have not produced a robust immune response. Part of the problem appears to be the low-level expression of the antigen that can be obtained, such that dosing would be variable, and regulatory agencies will not approve vaccines with variable dosing. Recent studies have, however, made significant progress in enhancing antigen expression and extraction in edible plants. Another concern that has been voiced regarding the development of edible vaccines using commonly eaten fruits or vegetables is the possibility that if the vaccine is presented together with the food in which it is produced, the intestinal immune system may view the vaccine as “food” and thus become tolerant. This would decrease rather than increase future response to invaders.

Storage of Vaccines—Strategies to Increase Shelf Life

One of the important requirements for an ideal vaccine is that it should be storable for long periods of time (i.e., it must be heat-stable, dry-stable, and have a long shelf life). This has been a very challenging property to satisfy. For those vaccine formulations that do not have long half-lives, it is often necessary to add stabilizers, such as adjuvants, emulsions, or gelatin (partially hydrolyzed pork, beef, or fish collagen), to protect the vaccine antigens from inactivation during freeze-drying or heating. People can develop allergic reactions against these stabilizers, increasing the risk of adverse vaccine-related side effects. For those vaccines that are difficult to sterilize, it is often necessary to add preservatives, such as mercuric thimerosal or antibiotics, to prevent microbial growth. Any mercuric thimerosal or antibiotics that are added during the manufacturing process must be removed from a childhood vaccine, which adds considerable cost to production. Residual quantities of any of these additives also increase the risk of adverse side effects or allergic reactions.

There is a major need for development of new vaccine formulations that are more stable with longer shelf lives and that do not require refrigeration. This is particularly critical for vaccines that are needed in developing countries, where storage conditions are less than optimal. Recent efforts in this arena include use of sugar-based **glassification**, which is a **lyophilization (freeze-drying)** process that includes vacuum drying with or without freezing. Glassification provides enhanced stability at room temperature. Other processes include CO_2-assisted nebulization with a bubble dryer that allows particles to be made in the range of 3–5 µm in size, which is desirable for lung delivery, without destroying biological activity of the vaccine.

Passive Immunization

The administration of antibodies obtained from immune animals or humans is an old solution to the problem of how to treat a patient who is not immune to a particular disease. This is called **passive immunization**. This type of therapy offers short-term protection to people who have recently been exposed to or soon may be exposed to that disease. Three types of passive immunization products are used. The first is a standard human immunoglobulin derived from pooled human serum that has been treated to kill viruses and contains primarily IgG against a broad spectrum of antigens. It has been used routinely to protect travelers from hepatitis A virus. It is now also a way to treat immunocompromised patients, especially those infected with common viral infections such as cytomegalovirus. In this case, preparations of immunoglobulin obtained from human sera are used because antibodies to cytomegalovirus antigens are commonly found in the sera of most people.

The second type is specific human immunoglobulin, which is derived from pooled human serum known to contain a high concentration of a specific antibody. This is a much more expensive reagent than the standard human immunoglobulin described previously. It is often used to treat patients recently exposed to hepatitis B virus.

The third type, which has been used for over 100 years, is animal-derived serum antibodies or antitoxins. The diphtheria and botulism antitoxins are prime examples of this type of passive immunization. In these cases, an animal (usually a horse) is immunized with a specific antigen, and then the antisera containing neutralizing polyclonal antibodies are harvested. A major problem with this type of product is serum sickness in about 10% of patients and occasionally death due to anaphylactic shock. One way to avoid this problem is to use human IgG antibodies acquired by pooling antisera from humans vaccinated with botulinum neurotoxin toxoid. Very little of this exceedingly expensive antitoxin is available, and so it is only used for rare cases of infant botulism. An exciting recent advancement in this area is the development of despeciated heptavalent botulism antitoxin, called H-BAT, which was licensed for commercial use in 2013 by the FDA, and neutralizes all seven A–G serotypes of botulinum neurotoxin. H-BAT is derived from despeciated horse IgG antibodies, which have the Fc portion of the antibody removed, leaving only the $F(ab')_2$ parts.

Passive immunization has been receiving much more attention recently as physicians finally admit that major human killers of the pre-antibiotic era, such as *S. pneumoniae,* the most common cause of bacterial pneumonia, have become so resistant to antibiotics that patients with multidrug resistant disease may no longer be treatable with antibiotics. Passive immunization becomes then even more important as a firewall against multiple antimicrobial-resistant microbes. Another treatment option is to provide antibodies that bind to the antiphagocytic capsule of this bacterium and enhance phagocytosis, thus destroying its ability to evade the nonspecific host defenses. More and more often, passive immunization is being invoked as a possible clinical response to multidrug resistant bacteria.

Passive immunization has also gained increased importance as the number of immunocompromised people has grown. This growing group of people not only includes those with HIV infections, but also those undergoing cancer chemotherapy or organ transplantation, and those living longer life spans. In these individuals who are unable to mount an effective immune response, passive immunization may well prove to be the only option for life-threatening infections. As the cost of vaccine development escalates and companies become more and more reluctant to pursue production of vaccines, passive immunization may well be the last line of defense against any disease for which there are no vaccines available.

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Questions

1. In the former Soviet Union, as it began to fall apart in the early 1990s, there was great unrest and population dislocation. Suddenly the incidence of diphtheria began to rise to alarming proportions. This might be understandable if all of the cases were in young children who might have fallen into the cracks of a compromised health system, which had been of high quality, at least in most areas, during the previous regime. But many of the cases were in adults. While it is still not clear exactly what happened, speculate on how the diphtheria vaccination program in the Soviet Union might have failed in the case of adults.

2. In 2004, there was an influenza vaccine crisis in the United States, when many lots of vaccine had to be recalled. The problem was bacterial contamination. Why would this be a problem with an injectable vaccine? Would it have been a problem with an oral vaccine? Under what conditions? There was only one major manufacturer of the vaccine, a fact that made the shortage worse. How could this shortage of manufacturers have happened?

3. It has been proposed that DNA vaccines might work better if the bacterial DNA were cloned into a viral vector that could integrate more efficiently into the human chromosome than naked DNA, as is done for gene therapy approaches. However, in the case of gene therapy using viral vectors, cancer has been a potential outcome.

How could this happen? Would this also be a problem for DNA vaccines? What could be done to prevent cancer from happening?

4. In this chapter, it was asserted that failures in vaccines could occur for three reasons. There are actually more. Can you think of some of them?

5. Most vaccines are equally effective in most populations. An exception to this rule was the tuberculosis vaccine, BCG. When administered to college students in the mid-1950s, it was very effective (about 80%) in preventing tuberculosis. In Bangladesh, the efficacy of the same vaccine was close to 0%. What are some of the reasons this could happen? Suppose that the vaccine was of high quality and the cold chain (refrigeration conditions) had been maintained but the disparity still occurred. Try to explain this. Hint (and this is highly speculative): there are bacteria related to *M. tuberculosis* that are found widely in the Indian subcontinent. How could this fact have affected the outcome? Remember, the percent effectiveness is the effectiveness above prevailing rates of TB.

6. How does passive immunization differ from active immunization? Why does passive immunization not confer long-term immunity? Under what circumstances might passive immunization be preferable to vaccination for treating infections?

7. What are the advantages and disadvantages of live vaccines? DNA vaccines?

8. Consider a bacterium that is ingested via contaminated water and locally infects the small intestine. What type of vaccine would be most useful against this bacterium? What type of immune response would this vaccine elicit?

9. How can recombinant DNA technology be used to enhance immunity and improve a vaccine?

10. What is a potential problem with use of diphtheria toxin as an antigen-delivery system? Can use of anthrax toxin overcome this problem?

11. Some scientists are looking into the strategy of using passive immunization to control bacteria that are resistant to many antibiotics. How would such a strategy work? (Recall that passive immunization is the injection of antibodies into a patient's bloodstream.)

12. Do vaccines necessarily work better if they target virulence factors than if they target molecules not involved in virulence? Explain why or why not.

13. Why might an IgG response to *Salmonella typhi* be ineffective?

14. Vaccination introduces _______, which mimics a particular pathogen, into the body, where it is engulfed by _________ that display pieces of it on the cell surface to other immune cells.

    a. an antigen/an antigen-presenting cell

    b. an epitope/an antigen-presenting cell

    c. a protein/an antigen-presenting cell

    d. an epitope/a dendritic cell

    e. a protein/a dendritic cell

15. _________ recognize and bind to epitopes on the surface of pathogens that have invaded the body and mature into an antibody-producing _________.

    a. Dendritic cells/naïve B cells

    b. Macrophages/B cells

    c. Naïve B cells/plasma cells

    d. Naïve B cells/B cells

    e. T helper cells/B cells

16. T helper cells send signals that encourage maturation of _________ into __________ that can locate, attack, and kill those host cells that are infected with a pathogen or are presenting an antigen.

    a. naïve T cells/cytotoxic T cells

    b. naïve T cells/mature T cells

    c. T helper cells/mature T cells

    d. T helper cells/cytotoxic T cells

    e. natural killer cells/cytotoxic T cells

17. To make a live vaccine it is important to make the pathogen ________.

    a. more virulent

    b. avirulent

    c. larger

    d. smaller

    e. less virulent

18. __________ are generated from inactivated bacterial toxins.

    a. All vaccines

    b. Subunit vaccines

    c. Recombinant vaccines

    d. Toxoid vaccines

    e. Conjugated vaccines

19. The usual length of time for vaccine development is ______.
    a. 1–3 years
    b. 2–5 years
    c. 5–10 years
    d. 10–15 years
    e. 15–20 years

20. Why are nonimmunized school-age children often protected from vaccine-preventable diseases, such as diphtheria, pertussis, measles, mumps, and polio?
    a. Because these children are still protected by maternal antibodies.
    b. Because the majority of children are immunized against these diseases.
    c. Because these diseases have been eradicated through global vaccination programs.
    d. Because these diseases exist nowadays only in underdeveloped countries.
    e. Because these children are not part of the demographic group targeted by these pathogens.

21. Smallpox was declared eradicated in 1980 by the World Health Organization (WHO). What does this mean and how does it differ from elimination of the disease?

22. Which is of the following is an accurate statement regarding the difference between active and passive immunity?
    a. Both are short-lived, but active immunity prevents disease symptoms right away.
    b. Both are long-lived, but passive immunity prevents disease symptoms right away.
    c. Active immunity uses one's own immune system to generate the antibodies that protect against the pathogen, whereas passive immunity uses antibodies generated by another host that are then used to protect against the pathogen.
    d. Active immunity uses antibodies generated by another host to prevent disease symptoms right away, whereas passive immunity allows for an immune response to slowly develop on its own.
    e. Active immunity uses antibodies generated immediately against the pathogen to prevent disease symptoms right away, whereas passive immunity allows for the immune response to develop on its own without any booster.

23. Which of the following is a correct statement with regard to an adverse event following immunization?
    a. An adverse event following immunization can be due to any adverse vaccine reaction caused or precipitated by a vaccine-related event when the vaccine is administered correctly.
    b. An adverse event following immunization can be due to an adverse vaccine reaction caused by errors in the administration of the vaccine.
    c. An adverse event following immunization can be due to an adverse vaccine reaction resulting from unrelated coincidence.
    d. An adverse event following immunization can be due to an adverse vaccine reaction due to the causes mentioned in A and B.
    e. An adverse event following immunization can be due to an adverse vaccine reaction due to the causes mentioned in A and C.
    f. An adverse event following immunization can be due to an adverse vaccine reaction due to the causes mentioned in B and C.
    g. An adverse event following immunization can be due to an adverse vaccine reaction due to the causes mentioned in A, B, and C.

24. Vaccines contain different components to make them effective; however, each component in a vaccine adds a potential risk of an adverse reaction. Which of the following is a list of common bacterial vaccine components that could contribute to the risk of an adverse reaction?
    a. egg proteins, latex, serum albumin, lactose, erythromycin, oil emulsion
    b. mammalian cell culture fluids, alum salts, collagen, thimerosal, paraffin emulsifier, formaldehyde
    c. egg proteins, polymyxin B, gentamicin, squalene emulsifier, mercuric thiomersal, cholesterol
    d. bacterial cell walls, glutaraldehyde, gelatin, mercuric thiomersal, oil emulsifiers, alum salts, gentamicin
    e. bacterial cell walls, penicillin, paraffin emulsifier, metal chelators, lactose, cholesterol

25. Determine whether the following statements are true or false.
    a. Killed or inactivated vaccines usually provide shorter length of protection than live vaccines.
    b. Vaccines are tested for safety in animals before they are used in clinical trials.

c. Vaccine manufacturers are responsible for issuing vaccine licenses in the United States.

d. Only those events that are caused directly by the vaccine components are reported to the Vaccine Adverse Event Reporting System.

e. The recommendation to receive a vaccine against pertussis as an adult is primarily to protect infants in the community.

f. Children with nonmedical vaccination exemptions are just as likely to contract certain vaccine-preventable diseases as children without those exemptions.

g. Vaccinations are not 100% effective in preventing disease.

h. Diseases that have animal reservoirs are more difficult to eradicate than diseases without them.

i. Passive immunization can lead to serious allergic reactions.

j. Although many of the first vaccines were live, attenuated vaccines, these types of vaccines are no longer used.

k. The United States has stockpiles of certain vaccines that can be used in the event of pandemic outbreaks of certain diseases.

Solving Problems in Bacterial Pathogenesis

1. A group of researchers has isolated a new bacterium W from the lymph nodes of several patients that returned from a camping trip and presented in the emergency room with high fever, rash, and swollen lymph nodes. The bacterium produces a unique surface protein (called OmpW) and an unusual polysaccharide capsule (called WPS) that the researchers can see on the surface of the bacteria in electron micrographs from fresh isolates (bacteria just obtained from the patients). However, after culturing the bacteria *in vitro* on agar plates, they find that most of the bacteria still have OmpW, but no longer have WPS on their surface. The researchers also find that bacterium W binds to and invades phagocytic cells and inhibits their function.

   a. The researchers propose that OmpW and WPS might make excellent targets for vaccine development against bacterium W. What led the researchers to propose this?

   b. The researchers find that a vaccine made of WPS alone does not evoke long-lasting immune responses. How does it elicit an immune response? What strategy could the researchers use to generate long-lasting immunity for bacterium W using WPS as part of the vaccine?

2. One of the alternative structures for the outer core of the lipooligosaccharide (LOS) found in clinical isolates of *Neisseria meningitidis* and *N. gonorrhoeae* is Galα[1-4]Galβ[1-4]Glc-, which has the same structure as globotriaosyl ceramide, Galα[1-4]Galβ[1-4]Glc-ceramide, a common glycolipid component of mammalian cell membranes. The loci encoding the biosynthetic genes for the outer core of LOS in *Neisseria* contain five conserved glycosyl transferase genes, *lgtA-E,* arranged in an operon. *lgtA, lgtC,* and *lgtD* contain poly-G tracts. The *lgtC* and *lgtE* genes encode the glycosyl transferases that connect the α-galactose and β-galactose, respectively, to the glucose of the outer core to form the Galα[1-4]Galβ[1-4]Glc- group.

   a. What would be the advantage for *Neisseria* to have Galα[1-4]Galβ[1-4]Glc- as part of its LOS? Be specific.

   b. One of the researchers in a vaccine development group at a biotechnology company proposes that Galα[1-4]Galβ[1-4]Glc- might make an excellent target for vaccine development against *Neisseria*. What might have led the researcher to propose this? Provide two possible reasons.

   c. Another researcher in the same vaccine development group challenged this proposal, stating that it was a poor choice for a vaccine candidate. What might have led the second researcher to propose this? Provide two possible reasons.

3. Both diphtheria toxin and anthrax toxin have been used as prototypes for development of novel vaccines and therapeutics.

   a. Describe how these toxins might be used as vaccines to deliver other antigens. What is the advantage of using these toxins as vaccine delivery vehicles?

   b. Diphtheria toxin was the first toxin to be exploited as an epitope-delivery vehicle. Many other toxins have since been used for this purpose. Name at least two problems with the use of diphtheria toxin as an epitope-delivery vehicle. What strategies have been employed to overcome these limitations? Name at least two.

   c. Describe how you might design a vaccine based on diphtheria toxin that might work to cure a tumor that overexpresses the ATR protein, which has an extracellular von Willebrandt factor A domain that binds to anthrax protective antigen, on the surface of the tumor cells.

4. The alarming spread of methicillin-resistant *Staphylococcus aureus* (MRSA) infection in the United States has prompted a number of research efforts toward development of a vaccine. MRSA is subcategorized as community-acquired (CA)-MRSA or hospital-acquired (HA)-MRSA, depending on how the infection is usually acquired. Although most CA-MRSA bacteria are not as resistant to antibiotics as the HA-MRSA strains, the recent strains that we are hearing about in the news appear to be more virulent. A group of researchers have recently discovered that bacterial cultures of a particularly virulent strain of CA-MRSA (called USA300) cause apoptosis in leukocytes and neutrophils, as evidenced by release of cytochrome c from mitochondria, followed by cytokine release and cell death. Most MRSA strains produce type CP5 or CP8 polysaccharide capsules, surface-exposed protein A, and a number of toxins and superantigens.

   a. Antibodies generated against bacterial culture medium from the USA300 strain identified two proteins of 33 and 44 kDa on Western blots. To determine the localization of these two proteins, researchers performed SDS-PAGE and Western blot analysis of lysates from the bacterial cell pellets and the bacterial culture medium as well as lysates of human neutrophils treated with the USA300 strain. The results from Western blot analysis are shown in the left panel of Figure 1. The researchers then applied the cell-free bacterial culture medium to the neutrophils and performed subcellular fractionation of the treated neutrophils. The results from Western blot analysis are shown in the right panel of the figure. Provide an interpretation (with rationale) of the results shown in Figure 1. Predict (with rationale) a possible function for the two proteins, which they have named LukS and LukF, where LukS is the 33-kDa protein and LukF is the 44-kDa protein. Provide at least one additional (different) experiment that could be performed to confirm your prediction. The researchers speculate that the two proteins, LukS and LukF, are two subunits of an AB-type toxin. Based on the results shown in Figure 1, do you agree with this interpretation? Provide your rationale. Provide an experiment that would allow you to verify your answer.

   b. Provide a mechanism for how protein A contributes to virulence (i.e., what is its function?).

   c. Provide a mechanism for how the superantigens contribute to MRSA virulence. What is a potential long-term consequence of infection with MRSA strains that produce superantigens?

   d. MRSA strains producing CP5 show a significantly higher bacteremia level than strains producing CP8. A group of researchers propose that development of a two-component vaccine made up of LukS or LukF in combination with CP5 or CP8 would be effective protection against CA-MRSA. What led the researchers to propose this? Provide at least two possible reasons. Which two of the possible vaccine targets (LukS, LukF, CP5, CP8) would be most effective at generating protective immunity as part of a two-component vaccine against CA-MRSA? Be sure to provide your rationale.

5. *Clostridium perfringens* is a Gram-positive anaerobic bacterium that can cause serious human and animal diseases, including clostridial myonecrosis (i.e., "flesh-eating" disease) and gas gangrene (i.e., "tissue-rotting" disease). *C. perfringens* strains are not intracellular pathogens, but are known to produce many extracellular enzymes and toxins, two of which are encoded by genes found in the chromosome of all pathogenic strains: perfringolysin O (PFO), a pore-forming cytolysin, and α-toxin, a zinc-dependent phospholipase C. These two toxins work synergistically during infection to cause gas gangrene and myonecrosis. You think it would be a great idea to develop a vaccine against *C. perfringens*. You propose that PFO and α-toxin would make excellent targets for development of a divalent component vaccine.

   a. Name two scientific arguments that you could provide to funding agencies to convince them to support

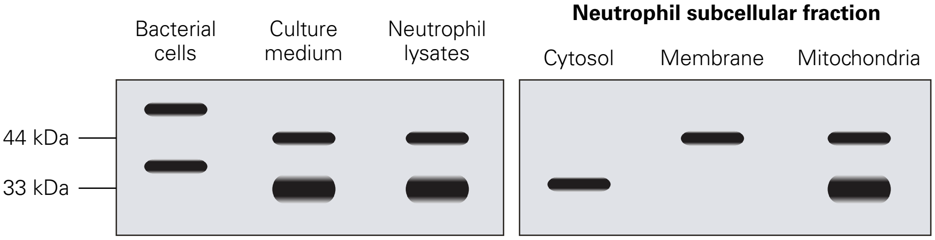

Figure 1. **Western blot results.**

your proposal. Include in your answer what type of immune response you are trying to achieve.

b. To test the effectiveness of these proteins as antigens, you use a vaccine formulation comprised of formalin-treated, heat-inactivated recombinant PFO and α-toxin mixed with alum as an adjuvant. You find that after immunization plus two boosters you get a strong immune response with high titers of antibodies against both proteins. However, in challenge studies using the mouse infection model, you find that there is no protection against sepsis with the *C. perfringens* strain, only protection from symptoms of gas gangrene and myonecrosis. Provide an explanation with rationale for these results.

c. Provide an alternative strategy (with rationale) for design of an optimal, safe recombinant vaccine based on PFO and α-toxin as antigens that does not rely on alum as an adjuvant, but will still lead to robust, long-lasting protection from symptoms of gas gangrene and myonecrosis by this pathogen.

Special Global Perspective Problems: Integrating Concepts in Pathogenesis

1. Lyme disease is the most common vector-borne disease in the United States. It is caused by the Gram-negative spirochete *Borrelia burgdorferi*, which is transmitted through the bite of infected ticks. The ability of the bacteria to survive in the tick and in mammals depends on the differential expression or repression of particular genes. For example, the bacteria express an outer surface protein A (OspA) in the midgut of nymphal and adult ticks, but down-regulate OspA and up-regulate another outer surface protein C (OspC) in response to increased temperature after the tick begins to feed on blood and the bacteria enter the host. Both OspA and OspC have become primary targets for vaccine development against Lyme disease. And, indeed, several commercial vaccines based on these proteins have been made and shown to have high efficacy in providing protective immunity in clinical trials.

a. Provide at least three reasons why OspA and OspC might be good vaccine candidates. Would OspA or OspC make a better vaccine candidate? State your rationale.

b. What type of immune response is primarily responsible for controlling *Borrelia* infection in healthy individuals who have been vaccinated against OspA or OspC? Be specific.

c. Describe how the immune response in an individual vaccinated with a vaccine based on OspC would function to control infection upon subsequent exposure to *Borrelia*. How would this differ from the response in an individual vaccinated against OspA?

d. Describe how *Borrelia* infection would be controlled during the first few days after exposure in healthy individuals who have NOT been vaccinated. Be specific. Provide the specific pathway(s).

e. Recent studies have determined that the immunodominant epitope in OspC responsible for protective immunity in humans is located in the highly conserved C terminus of the OspC. How is this OspC epitope presented to immune cells when it is used as a vaccine? Provide the specific pathway(s).

f. One consequence of disseminated *B. burgdorferi* infection in ~10% of infected individuals is a condition described as treatment-resistant Lyme arthritis (TRLA), which persists in patients even after antibiotic treatment. Recent studies have revealed that TRLA is correlated with a particular T cell epitope associated with rheumatoid arthritis, which has sequence similarity to a region of OspA spanning residues 165–173, which in turn is within a region of the protein that is important for providing protective immunity in humans. In clinical trials testing the efficacy of OspA as a vaccine candidate, this finding caused a serious problem. What was that problem? How could you correct it to improve the vaccine's safety, yet still retain its efficacy?

g. Studies aimed at designing diagnostic tests for Lyme disease have determined that animals, such as mice, hamsters, and dogs, do not react to OspC in the same way humans do. Indeed, animal models failed to produce immune responses to the dominant C-terminal epitope of OspC that is immunoprotective in humans. Consequently, the use of animal models to evaluate the efficacy and reliability of diagnostic tests for Lyme disease in humans has come into question recently. But, as a researcher, you are seeking to find additional diagnostic or vaccine targets against Lyme disease in humans. You would like to take advantage of the differential expression of proteins that occurs in *B. burgdorferi* when it is in the tick versus when it is in the human to identify such targets. Describe how you might accomplish this with no animal or human infection model available. Provide details of experimental design and rationale for choice of experimental approach.

2. Dental plaque is a biofilm consisting of a complex community of over 700 different bacterial species. Epidemiological evidence suggests that a population shift toward certain Gram-negative anaerobes is responsible for initiation and progression of periodontal diseases. *Tannerella forsythia*, a Gram-negative, filamentous, nonmotile, anaerobic bacterium, is considered one of the pathogens implicated in contributing to advanced forms of periodontal disease in humans and is strongly associated with cases of severe periodontitis. It is found coaggregated in periodontal pockets with other putative periodontal pathogens, such as *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Infection with *T. forsythia* induces alveolar bone resorption in a mouse infection model, involving inoculation under the gums of mice and then measuring for loss of dental bone. The organism is naturally resistant to gentamicin and erythromycin, but is sensitive to tetracycline, chloramphenicol, and kanamycin. The genome sequence of the bacterium is under way, but has not yet been completed and is still unavailable. Due to the fastidious (very picky) nature of this bacterium for growth (there are no auxotrophic mutants, and its growth on agar plates requires blood or hemin-supplemented amino acid-rich culture medium and incubation in an anaerobic chamber) and the difficulties in genetically manipulating it (there are no known transposons available, and the one *E. coli* shuttle vector reported thus far has very low conjugation frequencies and very poor transformation efficiencies), virulence factors that contribute to the role of *T. forsythia* in progression of periodontal disease have been difficult to identify.
   a. Considering all of the above information, which of Koch's postulates, if any, have been satisfied so far for *T. forsythia* involvement in periodontal disease? Be sure to state your rationale. Provide at least two additional modern molecular experiments (different from those already described) that could be performed to help satisfy Koch's postulates. (Be specific!)
   b. Considering all of the above information, describe a strategy that researchers who have a limited budget might use to identify virulence factors of *T. forsythia* that are associated with periodontal disease. Be sure to provide a rationale for your choice of strategy, the appropriate reagents that they will need to use for it (hint: a clearly labeled, detailed diagram or schematic of the overall strategy and/or tools to be used should be provided along with rationale for each of its features), the overall experimental design, and how the researchers might determine the identity of the putative virulence factors once found by this strategy.
3. Using your proposed screening strategy, the researchers have identified a number of putative virulence factors that they believe are involved in *T. forsythia* pathogenesis. Among these are several that have homology with proteins of known function:

   BspA: a fibronectin-binding cell-surface associated protein

   PrtH: a secreted trypsin-like protease

   SiaHI: a secreted sialidase

   HmuR: a hemin-binding outer membrane protein

   TfsA: a large 230-kDa surface (S-layer) glycoprotein with homology to *P. gingivalis* hemagglutinin

   a. What are the possible roles of each of these factors in *T. forsythia* pathogenesis? (Be specific!)
   b. What do these researchers have to do next to verify that these putative virulence factors are indeed involved in *T. forsythia* pathogenesis? Describe an experiment that could be performed to demonstrate this (which does not involve human subjects). What are the expected results (in terms of data) from such an experiment if these proteins are indeed virulence factors (i.e., provide a table, graph, plot, or gel illustrating this—you may make up example values or data points if that helps)?
4. Another group of researchers found that BspA and TfsA, in addition to having hemagglutination properties, are also involved in mediating bacterial binding and invasion of human epidermal cells.
   a. What type(s) of protective adaptive immune response does wild-type *T. forsythia* expressing these two proteins elicit? Be sure to provide your rationale.
   b. Describe in detail how these two antigens are presented to the immune cells in an unimmunized individual. (Hint: A detailed, clearly labeled schematic diagram might be helpful.)
   c. The researchers propose that BspA and TfsA might make excellent candidates for vaccine development against *T. forsythia*-associated periodontal disease. What led the researchers to propose this? Provide two different possible reasons.
5. The production of hemagglutinins is a well-established virulence strategy for a number of bacterial pathogens. Thus, it was not surprising that *P. gingivalis,* which is another putative dental plaque pathogen, also expresses several hemagglutinins on its cell surface. It was found that one of the hemagglutinin genes (*hagA*) of *P. gingivalis* contains four large contiguous direct repeats varying

from 1,318 to 1,368 bp in length, which together encode a protein of 283 kDa. The repeat unit (denoted as *HArep*), which contains the hemagglutinin domain, was also found to be present in the other hemagglutinin genes in *P. gingivalis* and in the *tfsA* gene (which encodes TfsA) from *T. forsythia*. The beginning amino acid sequence encoded by the first repeat (*HArep1*) in *hagA* is PNPNPGTTT, while that of the other three repeats (*HArep2-4*) is GTPNPNPNPNPGTTT. The amino acid sequence at the C terminus of the fourth repeat (*HArep4*) is GTPNPNPNP.

a. Provide an explanation that could account for the presence of this *HArep* repeat unit four times in *hagA* and also in the other hemagglutinin genes from *P. gingivalis* and in TfsA from *T. forsythia* (from a molecular evolutionary point of view).

b. Provide a possible mechanism for how this occurs. (Hint: A detailed, clearly labeled diagram or schematic might be helpful.)

6. Another group of researchers isolated a lipoprotein (which they named TfLP) from detergent extracts of *T. forsythia* bacterial cells that, when added to human oral fibroblast, epithelial, or monocytic cells, induced pro-inflammatory cytokine production (IL-1, IL-6, and TNF-α) and NF-κB–mediated apoptotic cell death. When this group of researchers submitted their manuscript reporting their findings to a scientific journal, one of the journal's reviewers rejected the manuscript because the researchers failed to include an important control experiment, stating that the cytokine production could have been due to a contaminating factor in the TfLP-containing bacterial extracts.

a. What factor did the reviewer think was a contaminant in the lipoprotein preparation and why was the possibility of this factor being present in the TfLP-containing extracts a concern? Be sure to provide your rationale.

b. If the researchers were to remove or ensure that this factor is not present in the TfLP-containing preparations, provide two possible mechanisms by which the TfLP lipoprotein might induce cytokine production and apoptotic cell death.

c. Describe how you might experimentally distinguish between these two possibilities.

18

IN THIS CHAPTER

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CHAPTER 18

The Gram-Positive Opportunistic Pathogens

It is easy to write off opportunistic pathogens, such as *Staphylococcus aureus* and *Clostridium difficile,* as being less important than overt pathogens, such as *Mycobacterium tuberculosis* or *Vibrio cholerae,* since they typically do not cause disease in healthy individuals with fully functional immune systems. However, at the end of the day, someone who died from a staphylococcal or clostridial infection is just as dead as someone who died from tuberculosis or cholera. In fact, in the developed world, despite all of the advances in modern medicine (and sometimes because of them), more people are killed by "opportunists" than by overt pathogens.

What Is an Opportunist?

The term "opportunistic pathogen" is used to describe microbial pathogens that usually do not cause disease in healthy people. However, when host defenses become compromised, opportunistic pathogens can cause serious damage. Compromised host defenses come in many forms, including breaches in innate barriers (e.g., burn wounds, cuts, or surgery), impairment of adaptive immunity (e.g., during HIV infection or after taking immunosuppressive drugs in conjunction with organ transplant or chemotherapy), or even genetic defects (e.g., individuals suffering from cystic fibrosis). However, there is a recent worrying trend for many of the traditionally opportunistic pathogens to infect seemingly healthy individuals with no identifiable underlying health conditions. Notable examples include *Staphylococcus aureus*, which causes a variety of diseases ranging from skin and soft tissue infections to endocarditis to toxic-shock syndrome, and community-acquired *Clostridium difficile*, which causes inflammatory colonic diseases. Furthermore, some opportunistic pathogens appear to be acquiring more dangerous virulence properties. For example, some strains of *Streptococcus pyogenes* cause relatively mild forms of disease such as superficial skin infections or pharyngitis (the so-called "strep throat"), but other, more "scary" strains of the same organism have received considerable press in recent years because of their "flesh-eating" properties.

What these cases may also illustrate is that within the average healthy human population there is a wide variation in susceptibility to different infectious diseases. Looking at things from the "overt pathogen" side, it is worth noting that even in the case of pathogens, such as *Vibrio cholerae* (the cause of cholera), *Salmonella enterica* serovar Typhi (the cause of typhoid fever), and *Mycobacterium tuberculosis* (the cause of tuberculosis), there are often cases where infected individuals are asymptomatic. Consequently, the picture of host-pathogen interactions is evolving more toward a view that there are probably no hard and fast distinctions between "opportunists" and "overt pathogens," but rather a continuum over a wide range of outcomes, with some skewing toward disease in seemingly healthy individuals and others toward more severe disease in individuals with clear underlying conditions. Nonetheless, the term "opportunist" has proven useful as a general concept and will be used here and in chapter 19, where Gram-negative opportunists will be covered.

Characteristics of Gram-Positive Opportunists

A common feature of the Gram-positive opportunists featured in this chapter is that they are all normal commensal inhabitants of the human body. Bacteria that reside on the human body have a significant advantage when it comes to causing disease in that they are often present in large numbers at the sites of potential breaches in host defenses. Once these bacteria reach the blood stream, Gram-positive pathogens, like their Gram-negative counterparts, trigger sepsis (see chapter 3). The longer sepsis persists, the more likely an infected person will suffer long-term damage to essential organs or death. Unfortunately, treatment is often made difficult by the high prevalence of many different forms of antibiotic resistance in opportunistic pathogens. As a result, when these opportunists manage to cause an infection, the infection often ends up being very difficult to treat.

Given the seriousness of infections by opportunists, an important question to consider is how are commensal bacteria able to cause invasive diseases in the first place? It turns out that the answer depends largely on the specific organism in question. The distribution of different bacteria varies widely among the human population, with some bacteria colonizing everyone all of the time, and others colonizing only a portion of the population at any given time. Scientists today are actively investigating the various genetic and environmental factors that lead to this variability. Aside from differences in the distribution of bacterial populations, different isolates of ostensibly the same bacterial species can cause a wide spectrum of different diseases ranging in severity from mostly innocuous to tragically fatal. Recent genomic, transcriptomic, and proteomic comparisons have revealed that each of these species is actually composed of several distinct subgroups or strains with profound differences in their genomes and the virulence factors they carry. Ultimately, each species is comprised of a conserved core set of genes, referred to as the **core genome**. In any individual isolate, the core genome typically only accounts for roughly 80% of its genome. The remaining 20% is comprised of various genomic islands, prophages, plasmids, or mobile genetic elements that are not shared among all strains. These "accessory" genes often carry distinct sets of virulence factors that determine whether a particular strain acts as a well-behaved commensal or as an invasive disease-causing pathogen. The core genome, along with the set of all accessory genes from all strains of a given species, are collectively referred to as the **pangenome.** This notion of a pangenome has led to new ways of identifying and categorizing bacterial subspecies.

Notable Gram-Positive Opportunists

For this section of the chapter, we will focus on four representative examples of Gram-positive opportunists: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, and *Clostridium difficile*. These examples were chosen to provide a framework for thinking about the diverse factors and mechanisms used by Gram-positive opportunists to exist as innocuous components of our microbiota and to cause potentially devastating disease as invasive pathogens. As we go through the different features of each organism, hopefully their similarities and shared mechanisms will also become evident.

Staphylococcus aureus—Commensal Ready for a Fight

The name *S. aureus* (Latin: *aureus* = "golden") comes from the golden color of the colonies it forms on rich medium. A classical defining characteristic that separates *S. aureus* from other *Staphylococcus* species is the presence of an enzyme called **coagulase**, which catalyzes the conversion of fibrinogen to fibrin, which results in blood clotting. Coagulase is easily detected by a simple clinical test. *S. aureus* is also known for its durability in the environment. At least one-third of the human population is colonized by *S. aureus* in the nose (mostly in the anterior nares, including the nostrils) and the upper respiratory tract. Many people are persistent, asymptomatic carriers of *S. aureus*, while about an equal number are colonized only transiently. These asymptomatic carriers have a higher risk of infection than noncarriers and can serve as reservoirs for person-to-person spread. Furthermore, although the main human site for *S. aureus* colonization is the nose, *S. aureus* also sheds onto the skin and into the environment, where it can last for long periods of time. Together, these properties make *S. aureus*, particularly its multidrug resistant strains like methicillin-resistant *S. aureus* (MRSA), extremely problematic for health care facilities. Indeed, MRSA has become a leading cause of health care-associated and nosocomial infections.

Understanding where *S. aureus* lurks has been instrumental in the development of strategies for limiting the nosocomial spread of these bacteria. For example, because *S. aureus* resides on skin surfaces and in the nose, topical antibiotics like mupirocin that are too toxic for internal use can be effective against *S. aureus*. Consequently, the application of mupirocin to the noses of colonized health care workers can decolonize these workers, thereby defusing the proverbial ticking infectious disease time bomb. Similarly, because *S. aureus* survives on articles of clothing like neckties, even though hospital gowns and gloves appear to be

Box 18-1.
An Unexpected Deadly Outcome for a Convenient Superabsorbent Innovation

In 1978, a new brand of tampons was marketed as having a revolutionary new superabsorbent design allowing them to stay in the vagina for extended periods of time. Unfortunately, this new design also resulted in a perfect storm of multiple factors to create an ideal environment for *S. aureus* to grow and cause deadly disease.

First of all, being superabsorbent in itself was problematic in that the tampon could dry out the vaginal cavity resulting in tearing of the vaginal lining when the tampon was removed, effectively breaching the first line of innate host defenses. Second, the absorbent material of the tampon was made using a synthetic gelling agent and foam cubes. After absorbing moisture, the gelling agent acted like petri dish agar to provide a viscous medium, while the foam cubes provided ample surface area for the bacteria to grow. Additionally, this foamy material also enabled air into the normally anaerobic vaginal cavity, which further enhanced the ability of *S. aureus* to grow.

In addition to the design of the tampon, other factors that were not considered exacerbated the problem. For example, the pH of the vagina is normally fairly acidic due to the presence of an abundance of lactic acid-producing *Lactobacillus* species (see chapter 5), which keeps many bacteria, including *S. aureus*, in check. However, during menstruation, the pH actually rises to around neutral pH, which is ideal for expression of TSST-1. Additionally, the release of TSST-1 and other toxins induces fever, and the elevated temperature is beneficial to *S. aureus* growth.

At the time, this type of infection was completely unheard of with regard to *S. aureus*. Even though *S. aureus* was known to exist on the body, it was mostly considered to be inert. In fact, during product testing, the primary focus was on the safety of the constituent components of the materials used in the tampon, rather than potential changes in the vaginal microbiota. Ultimately, this case drastically changed the way scientists, product manufacturers, government regulators, and the public at large think about our bodies and its response to exposure to various materials. In determining the safety of a product, particularly one that is placed on or inside our bodies, one must consider not only the interaction of the product with our body, but also how it interacts with the microbes that reside in us.

As might be expected, once this superabsorbent tampon was recalled and removed from the market, cases of toxic shock syndrome caused by *S. aureus* virtually disappeared with fewer than one case per 100,000 people per year occurring in recent decades.

Source:

Vostral SL. 2011. Rely and toxic shock syndrome: a technological health crisis. *Yale J Biol Med* **84**:447–459.

the main vector for transmission between patients, doctors are being asked to abandon clothing accessories like scarves and ties when possible as a safety precaution.

A disturbing trend associated with the emergence of community-associated (CA)-MRSA strains seems to be their spread in the absence of nasal colonization, probably from colonization sites on the skin. As the name implies, strains of CA-MRSA, which cause severe necrotizing pneumonia and contagious skin infections, are not confined to hospitals and are often acquired from contact in schools, recreational centers, nursing homes, and other community settings. Once *S. aureus* gains access to tissues via breaches of barrier defenses, it employs a number of adhesins for binding to extracellular matrix proteins and recruits PMNs (see chapter 3) to the infection site. Then, through the release of activating and inhibiting cytokine modulators, it mediates the formation of **abscesses** (Figure 18-1). The increasing prevalence of CA-MRSA is likely due to changes in the expression and functions of some of the bacterial virulence factors discussed below.

Toxins. Different *S. aureus* strains can cause a variety of toxin-mediated diseases, including skin infections, such as impetigo and boils. They also can colonize plastic implants, leading to endocarditis. *S. aureus* produces two classes of superantigens: **toxic shock syndrome toxin 1** (**TSST-1**) and **staphylococcal enterotoxin** (**SE**), of which there are at least seven types: SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE (see Table 12-1). As we discussed in chapter 12, superantigens are proteins that force an association between major histocompatibility complex II (MHCII) on antigen-presenting cells and T-cell receptors that would not normally occur (Figure 12-4). This association bypasses the need for presentation of specific immunogenic epitopes and is tight enough to trigger cytokine release by both cell types. When these complexes form, excess cytokine is released and triggers **toxic shock syndrome**. Fever, rash, and sloughing skin are some of the early symptoms, and if not successfully treated, death can eventually ensue. See Box 18-1 for an account of toxic shock syndrome caused by *S. aureus* that first made headlines in the late 1970s.

Enterotoxin-producing *S. aureus* strains are a major cause of foodborne disease (or food poisoning). SE is ingested with food, and once in the stomach, it stimulates the vagus nerve endings, which control the vomiting reflex that results in projectile vomiting and abdominal pains. Consistent with SE-mediated disease being an intoxication rather

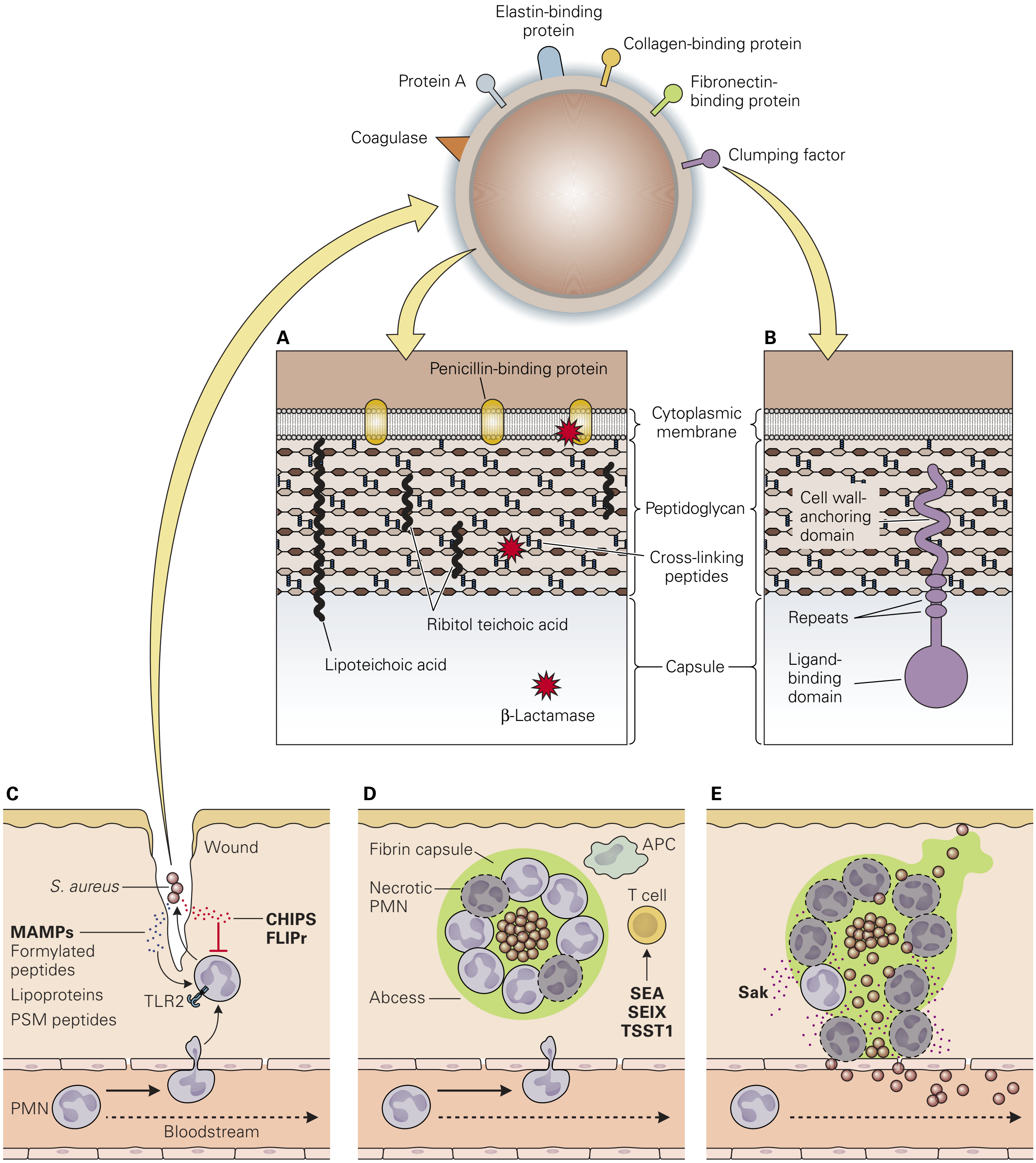

Elastin-binding protein
Collagen-binding protein
Protein A
Fibronectin-binding protein
Coagulase
Clumping factor
A
B
Penicillin-binding protein
Cytoplasmic membrane
Peptidoglycan
Cross-linking peptides
Capsule
Ribitol teichoic acid
Lipoteichoic acid
β-Lactamase
Cell wall-anchoring domain
Repeats
Ligand-binding domain
C
D
E
Wound
S. aureus
MAMPs
Formylated peptides
Lipoproteins
PSM peptides
TLR2
CHIPS
FLIPr
PMN
Bloodstream
Fibrin capsule
Necrotic PMN
APC
T cell
Abcess
SEA
SEIX
TSST1
Sak

Figure 18-1. Virulence factors of *Staphylococcus aureus* associated with stages of infection and abscess formation. *Staphylococcus aureus* displays on its surface or secretes a number of virulence factors that promote colonization and dissemination in the body. **(A)** The cell wall is comprised mainly of peptidoglycan consisting of a polysaccharide chain of alternating N-acetylglucosamine and N-acetylmuramic acid residues with 1,4-β linkages. The chains are crosslinked tetrapeptide chains attached to certain N-acetylmuramic acid residues and bridged by a pentaglycine chain. Ribitol teichoic acids are also attached to the peptidoglycan. Lipoteichoic acids (LTA) are linked to a glycolipid at one end that anchors them in the cytoplasmic membrane, where penicillin-binding proteins are also localized. **(B)** Many surface proteins have structural features that are similar to that of clumping factor, including an N-terminal secretory signal sequence, a cell-wall-anchoring domain and repeated amino acid sequences that span the peptidoglycan and capsule, and a C-terminal ligand-binding

than a bacterial infection, symptoms usually appear within a few hours of eating contaminated food, and usually completely subside after a day or two.

The *tst, entA*, and *entB* genes, encoding TSST-1, SEA, and SEB, respectively, are carried on temperate bacteriophages, but other SE genes (e.g., *entC* and *entD*) are carried on plasmids (see Table 12-1). As we alluded to earlier in the chapter, the prevalence of superantigen genes in *S. aureus* challenges any strict definition of opportunist. After all, if opportunistic infections are simply the result of a breach in host defenses, why do *S. aureus* strains maintain their various superantigens? It has been speculated that superantigens might contribute to transmission in humans or other hosts, but the precise role of the superantigens in the biology of *S. aureus* remains unclear.

In addition to superantigens, there are several other toxic staphylococcal exoenzymes that contribute to the survival and spread of the bacteria in the human body. For example, a group of proteases, called **exfoliative toxins (ETs)**, are produced by some *S. aureus* strains and cause skin conditions. In infants, the disease scalded skin syndrome gets its name from the red peeling skin infected infants develop, which looks similar to a bad scald. These same strains have also been implicated in bullous impetigo, a type of skin infection that produces blister-type lesions. In both cases, ETs cause exfoliation, or separation of skin layers. The best-studied ET is exfoliative toxin A, which specifically cleaves desmoglein-1 (Dsg-1), a cadherin protein required for maintaining keratinocyte cell-cell adhesion in the basal layers of the epidermis. When Dsg-1 is cleaved, skin keratinocytes separate, causing the epidermis to separate from the underlying tissue.

Another exoenzyme contributing to bacterial spread is **staphylokinase (Sak)**, which dissolves blood clots by effectively deregulating the normal process by which the body breaks down blood clots. Blood clots are made up of platelets held together by a fibrin mesh. One of the components of this mesh is the protein **plasminogen**. Normally, during dissolution of a clot, endothelial cells secrete a serine protease, called tissue plasminogen activator, which cleaves plasminogen to an active form, called **plasmin**. Plasmin itself acts as a serine protease that degrades the fibrin mesh, thereby dissolving the clot (Figure 18-2). This is a carefully controlled process that is confined to the clot as free plasmin in the bloodstream is rapidly degraded. Sak forms a 1:1 complex with plasminogen effectively activating its latent protease activity to cleave other plasminogen molecules into plasmin in an uncontrolled way. This uncontrolled release of plasmin destroys the extracellular matrix and fibrin fibers that hold cells together, allowing the bacteria to move through tissue and to escape from abscesses (see Figure 18-1). Interestingly, similar to some of the toxins discussed in chapter 12 for which pharmaceutical or cosmetic applications have been developed, **streptokinase**, a homolog of Sak found in several different β-hemolytic *Streptococcus* species, is used therapeutically as a thrombolytic agent to dissolve blood clots.

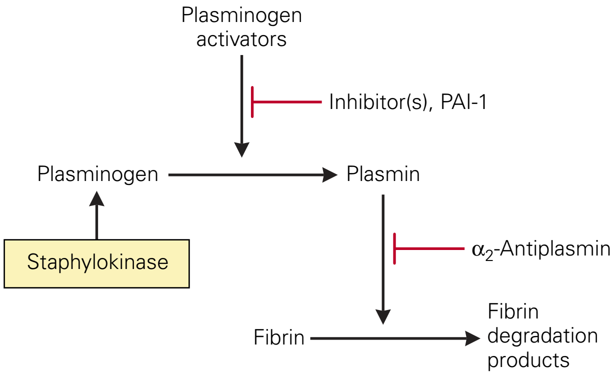


Figure 18-2. Staphylokinase activation of the fibrinolytic system to dissolve blood clots. Normally, activators and inhibitors in the blood carefully control the clotting cascade process. Staphylokinase (Sak) binding to plasminogen leads to uncontrolled cleavage of other plasminogen molecules to plasmin, resulting in fibrin degradation, which leads to dissolving of blood clots and spread of the bacteria. PAI, host plasminogen activator inhibitor.

Other staphylococcal exotoxins function to kill or limit the ability of immune cells to attack the bacteria. A β-barrel, pore-forming exotoxin, called **α-toxin**, forms pores in human cell membranes and serves as a defense against neutrophils and other immune cells. This toxin used to be called

Figure 18-1. *continued*

domain. **(C)** Once the bacteria obtain access to tissues through open wounds, they use adhesins, such as fibrinogen-binding proteins A (FnBPA) and B (FnBPB), iron-regulated surface determinant protein A (IsdA), clumping factors A (ClfA) and B (ClfB), and collagen adhesin (Can), to attach to extracellular matrix proteins, such as fibronectin, cytokeratin 10, and collagen. Bacteria produce phenol-soluble modulin (PSM) peptides that promote release of proinflammatory lipoproteins (a kind of microbe-associated molecular pattern; MAMP), which activate TLRs and lead to cytokine production and local inflammation. The bacteria recruit and modulate PMNs through small modulating peptides, such as formylated peptides and PSM, and inhibitors of PMN chemotaxis, such as chemotaxis inhibitory protein from *Staphylococcus* (CHIPS) and FPRL1, inhibitory protein (FLIPr), and this modulation plays a role in abscess formation. **(D)** The bacteria produce coagulases that polymerize fibrin and form the abscess around the bacteria at the infection cite. The bacteria also release pore-forming toxins, such as leukocidins (Panton-Valentine leucocidin, PVL) and α-toxin that lyse PMNs; superantigens (TSST-1, SEA, SEIX) that stimulate inflammation by nonspecific T-cell activation; and other virulence factors, such as polysaccharide microcapsule (PGA), surface protein A (SpA), and IgG-binding protein (Sbi), that prevent opsonization and killing by PMNs. **(E)** The bacteria can escape from the abscess and be released into the bloodstream through the action of plasminogen-activating protein staphylokinase (Sak). Adapted from Lee AS, de Lencastre H, Garau J, Kluytmans J, Malhotra-Kumar S, Peschel A, Harbarth S. 2018. *Nat Rev Dis Primers* **4**:180333, with permission.

α-hemolysin (Hla) because it lyses red blood cells, as well as other cell types. Membrane damage by α-toxin triggers cytokine production and sets off the apoptotic pathways that kill damaged cells. It has been implicated in some of the necrotizing pneumonia cases caused by CA-MRSA strains. Some strains of *S. aureus* produce other hemolysins: β-hemolysin, δ-hemolysin, and γ-hemolysin, which can damage membranes of cells other than red blood cells and, similar to α-toxin, may contribute to reducing the immune response.

The **Panton-Valentine leucocidin (PVL)** is another extracellular pore-forming cytotoxin that damages mammalian cell membranes. PVL is associated with most CA-MRSA isolates, though its role in virulence remains unclear. Its name arises from the fact that it can kill leukocytes, including neutrophils, macrophages, and lymphocytes. PVL consists of two protein components, LukS and LukF, that assemble to form large pores in membranes (Figure 18-3). It has been shown that all three proteins responsible for γ-hemolysin activity (HylA, HylB, and HylC) have amino acid sequences nearly identical to sequences of LukS and LukF.

Other virulence factors. *S. aureus* strains produce a variety of other factors involved in colonization and virulence. One important class of factors mediate attachment of *S. aureus* cells to certain blood proteins, such as IgG and fibrinogen, or to extracellular matrix proteins, such as fibronectin and collagen. The surface adhesins that bind to extracellular matrix proteins have been given the acronym **MSCRAMMs**, which stands for **microbial surface components recognizing adhesive matrix molecules**. Several MSCRAMMs are involved in colonization and virulence and allow the bacteria to adhere and persist in the host. These MSCRAMMs include fibronectin-binding protein, collagen-binding protein, laminin-binding protein, vitronectin-binding protein, and elastin-binding protein (see Figure 18-1, and also Figure 11-4). Close proximity of *S. aureus* to the extracellular matrix provides a rich mixture of host proteins and polysaccharides. *S. aureus* produces surface enzymes that digest and dissolve these components of the extracellular matrix, providing food for the bacteria, exposing additional attachment sites for bacterial adherence, and increasing the chances for the bacteria to spread through tissues. The MSCRAMM called **clumping factor**, which binds to fibrinogen in blood clots and can cause clumping of human plasma, has an additional role in protecting *S. aureus* from the immune system. Clumping factor binds to serum complement factor I that stimulates the cleavage of complement C3b fragment on the *S. aureus* surface to its inactive (iC3b) form. This inactive form cannot associate with factor B and therefore cannot amplify the complement cascade, effectively reducing phagocytosis of the bacteria by neutrophils. For more details on complement, see chapter 3.

Most MSCRAMMs and enzymes that digest host proteins and polysaccharides are covalently attached to the bacterial peptidoglycan cell wall by **sortases** (Figure 18-4A, see also

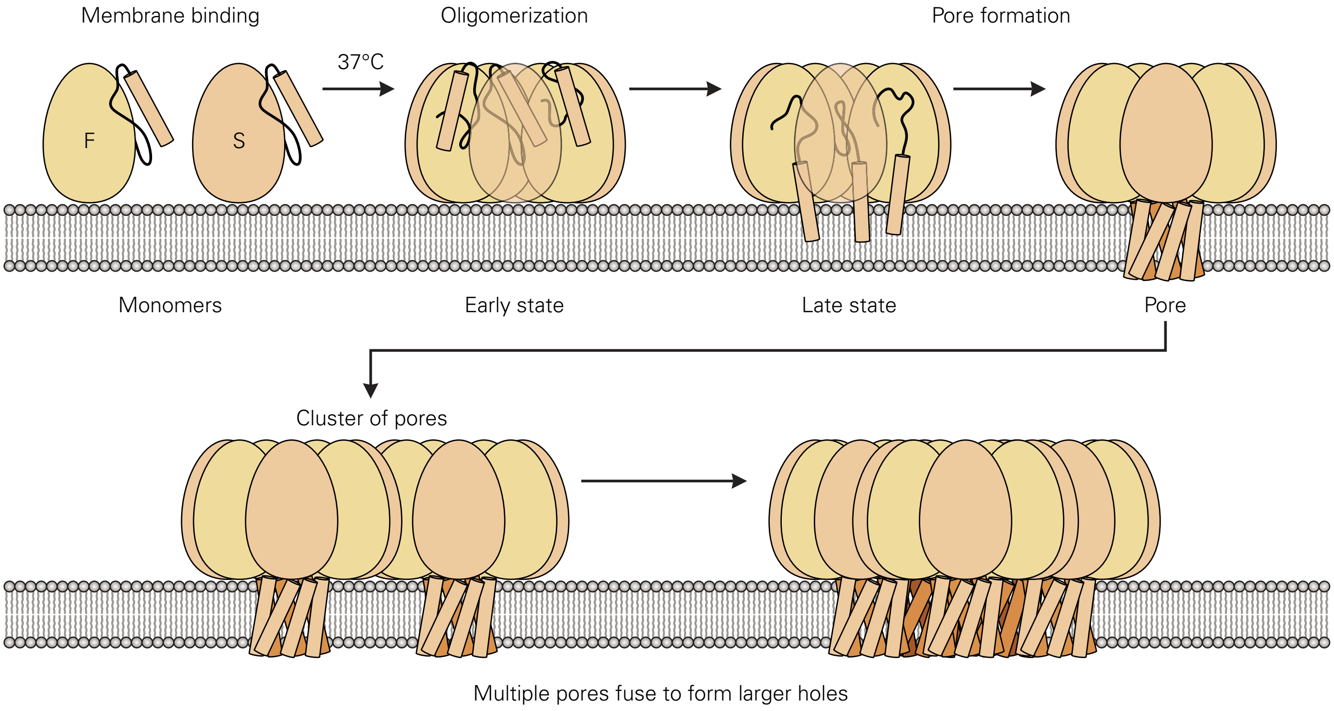


Figure 18-3. Steps in the pore formation by the two-subunit toxin, leukocidin. Monomers of LukS (S) and LukF (F) bind to GM1 gangliosides and assemble on the surface of host cells. Upon formation of a heptamer, the stem-loop undergoes a conformational change and inserts into the plasma membrane to form the pore. Multiple pores can cluster to form larger holes in the membrane.

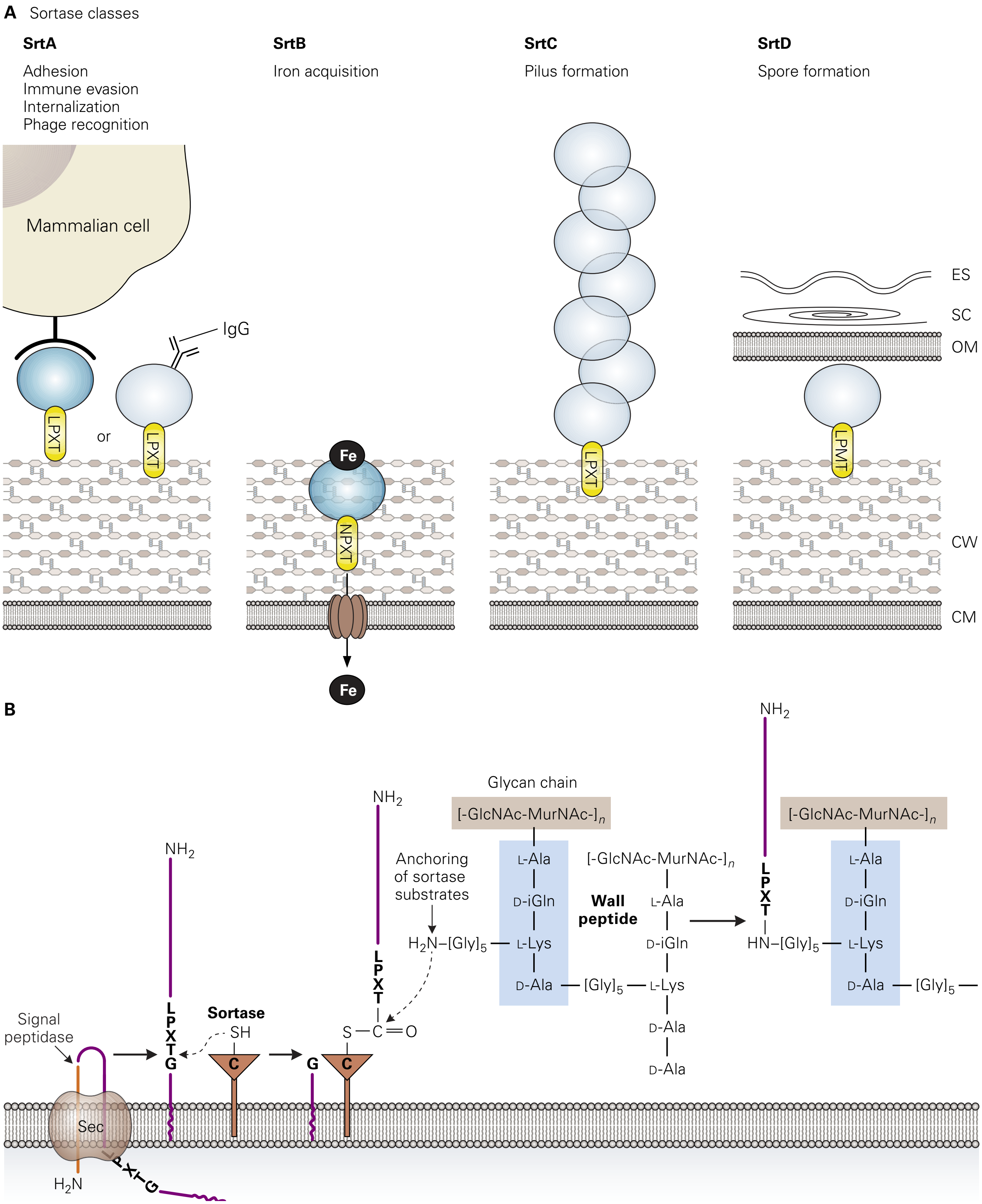

A Sortase classes
SrtA
Adhesion
Immune evasion
Internalization
Phage recognition
SrtB
Iron acquisition
SrtC
Pilus formation
SrtD
Spore formation
Mammalian cell
IgG
LPXT
or
LPXT
Fe
NPXT
Fe
LPXT
LPMT
ES
SC
OM
CW
CM
B
Signal peptidase
Sec
H2N
LPXTG
NH2
LPXTG
Sortase
SH
C
G
C
NH2
LPXT
S—C═O
Anchoring of sortase substrates
H2N–[Gly]5
Glycan chain
[-GlcNAc-MurNAc-]n
L-Ala
D-iGln
L-Lys
D-Ala
[Gly]5
[-GlcNAc-MurNAc-]n
L-Ala
D-iGln
L-Lys
D-Ala
D-Ala
Wall peptide
NH2
LPXT
HN–[Gly]5
[-GlcNAc-MurNAc-]n
L-Ala
D-iGln
L-Lys
D-Ala
[Gly]5

Figure 18-4. Four different classes of Gram-positive bacterial sortases based on sequence homology and cellular function. (A) Class A sortase, SrtA or housekeeping sortase, is responsible for the cell wall anchoring of proteins that are involved in bacterial adhesion, immune evasion, internalization, or function as receptors for phage binding. Class B sortase, SrtB, anchors proteins to the cell wall envelope that are specifically involved in iron acquisition. Class C sortase, SrtC, assembles pili on the surface of Gram-positive bacteria. Class D sortase, SrtD, anchors proteins to the cell wall peptidoglycan as the bacilli (or *Streptomyces*) engage in sporulation, a developmental program that generates dissimilarly sized daughter and mother cells. Recognition motifs for each class of sortase are highlighted in yellow. CM, cell membrane; CW, cell wall; OM, outer membrane; SC, spore coat; ES, exosporium. **(B)** The cell wall of *S. aureus*. The repeating disaccharide *N*-acetylmuramic acid-(1–4)-*N*-acetylglucosamine (GlcNAc-MurNAc) is amide-linked to the alanine of the pentapeptide [L-Ala-D-iGln-L-Lys-(Gly5)-D-Ala]. Its pentaglycine cross bridge is linked to the carboxyl group of D-Ala at position 4 of a neighboring cell wall tetrapeptide. The amino group of pentaglycine cross bridges is also the site of sortase-mediated anchoring of surface proteins (arrow). Based on Maresso AQ, Schneewind O. 2008. *Pharmacol Rev* **60:**128–141.

Figure 11-10). In *S. aureus*, the muropeptide chains in the peptidoglycan are linked by characteristic pentaglycine cross bridges (Figure 18-4B). The class A "housekeeping" sortase recognizes secreted proteins containing the sortase recognition motif, LPXTG, and then cleaves the T–G peptide bond to form a covalent acyl-enzyme intermediate. The free N terminus of an unlinked pentaglycine bridge then displaces the sortase to create a new peptide bond, attaching the secreted protein to the peptidoglycan cell wall. Different Gram-positive bacteria contain additional classes of specialized sortases that each recognize slightly different recognition motifs. *S. aureus* contains a general sortase that attaches numerous MSCRAMMs and surface proteins as well as more specific sortases used to attach pili and other proteins involved in heme uptake and iron acquisition. Gram-positive species that form spores, such as *C. difficile* (discussed in more detail below), also contain a specialized sortase for assembly of the spore coat. Since sortase-attached proteins play such critical roles in colonization and virulence, the sortase enzyme has become a possible target for the development of new antibiotics.

Given the exposed nature of the surface adhesins and their putative roles in virulence, they might seem like good targets for the immune system. However, despite producing antibodies against these surface proteins, people and animals infected with *S. aureus* typically do not become immune to reinfection. Similarly, development of a *S. aureus* vaccine has been largely unsuccessful despite several vaccines eliciting high antibody titers in vaccine recipients during clinical trials. One explanation suggested for this is the production of antibodies against the staphylococcal **fibronectin-binding protein (FnBP)**. These antibodies do not block binding of the FnBP to fibronectin, but rather, they bind to FnBP after it has already attached to fibronectin.

There are a number of factors that contribute to the resilience of *S. aureus* against the adaptive immune system. One of particular interest is **Protein A**, which is anchored to the cell wall by sortase and binds to the Fc portion of IgG antibodies (Figure 18-5, see also Figure 11-4). For an important biotechnological application that exploits this binding property of protein A, see Box 18-2. This binding of protein A to the Fc portion prevents IgG from binding to receptors on phagocytic cells, thereby preventing opsonization of the *S. aureus* bacteria. Furthermore, protein A is also able to bind to the antigen-binding Fab domain of the **membrane immunoglobulin (mIg)** that is part of B-cell receptor complexes (see chapter 4). This binding then triggers a cellular response pathway culminating in programmed cell death, effectively depleting the B cell population from an infected host. As a result, protein A deals a 1-2 punch, first preventing antibodies from properly binding to the *S. aureus* bacteria, then preventing the immune system from mounting further antibody responses.

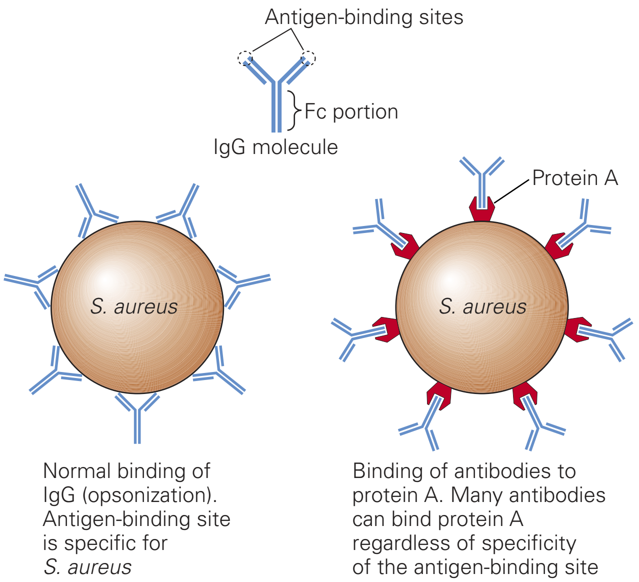

Antigen-binding sites
Fc portion
IgG molecule
Protein A
S. aureus
S. aureus
Normal binding of IgG (opsonization). Antigen-binding site is specific for S. aureus
Binding of antibodies to protein A. Many antibodies can bind protein A regardless of specificity of the antigen-binding site

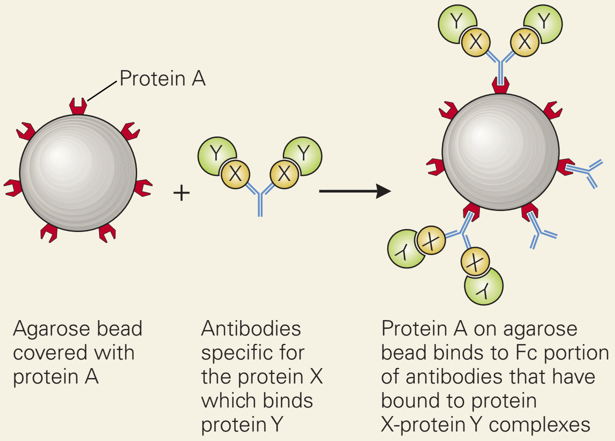
Figure 18-5. Binding of IgG by protein A. Protein A binds to IgG through the Fc region (right) rather than the antigen-binding sites in the normal way for antibody binding to an antigen for opsonization (left).

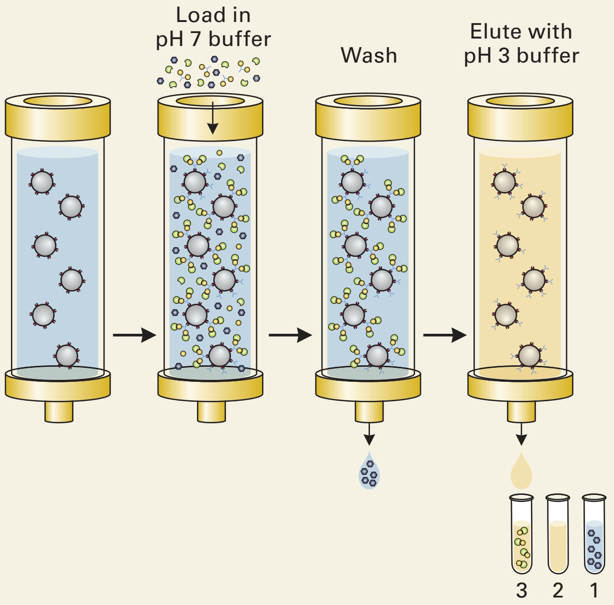
Because *S. aureus* is found almost exclusively outside of cells in pathology specimens, it was originally believed to be a strictly extracellular pathogen. However, it is now classified as a facultative intracellular pathogen, given its ability to escape the phagosome and live and grow within several different host cell types. These include endothelial cells, which may facilitate transit of bacteria from tissues to the bloodstream and dissemination in the body. This intracellular behavior likely contributes to the persistence of *S. aureus* infections and frequent relapse after seemingly successful treatment of staphylococcal infections, as these intracellular bacteria are protected from standard antibiotic treatments and may function as a reservoir for future infection.

Antibiotic resistance. Although we covered antibiotic resistance more thoroughly in chapter 15, it is impossible to discuss *S. aureus* without mentioning antibiotic resistance, given the significant epidemiological burden posed by the growing prevalence of virtually untreatable multidrug-resistant *Staphylococcal* infections. The first report of penicillin resistance in *S. aureus* was published about a year after the first use of penicillin to treat human infections (see chapter 15 for the mechanism by which penicillin kills bacteria), though it is likely that these resistant strains were around even earlier. Since then, strains of *S. aureus* have become resistant to more and more antibiotics. Now, there are some strains that are only treatable with the so-called "drugs of last resort," such as vancomycin or more recently, a combination of streptogramins and linezolids (these antibiotics are covered more thoroughly in chapter 15). Unfortunately, *S. aureus* resistance to linezolid was reported only about one year after the drug was first introduced in 2000.

Box 18-2.

Exploiting Bacterial Protein A and Protein G for Affinity Chromatography Applications


Protein A
+
Y
X
Agarose bead covered with protein A
Antibodies specific for the protein X which binds protein Y
Protein A on agarose bead binds to Fc portion of antibodies that have bound to protein X-protein Y complexes



Scientists have exploited the IgG-binding property of staphylococcal protein A for biotechnological and biomanufacturing applications related to protein purification or detection of specific proteins for diagnostic testing. The protein A is attached to chromatography resin beads and then IgG, raised against a particular protein of interest, is bound to protein A through its Fc region. Similarly, protein G from group C or G streptococcal species can be conjugated to agarose beads and used to harvest IgG that is bound to other proteins through the antigen-binding sites. The choice of protein A or protein G depends on the class of IgG antibody that is being immobilized on the bead. The affinity of protein A or protein G for antibodies varies considerably among human and animal species. Protein A binds with high affinity to human IgG1, IgG2, and IgG4, but poorly to IgG3, whereas protein G binds to all four IgG isotypes. Protein A, but not protein G, also binds to human IgA, IgM, and IgE.

The IgG-coated beads can be mixed with cell lysates or other complex mixtures containing the protein of interest. The beads can then be separated back out of the solution through low-speed centrifugation or sedimentation, taking the protein of interest with them. The beads can be washed to remove unbound proteins, and the bound protein of interest can then be stripped from the IgG-coated beads, thereby affinity purifying the proteins recognized by the antibodies.

The immobilized IgG can also be used to capture proteins from complex mixtures. This process is called protein **immunoprecipitation (IP)** or is referred to as a **pulldown assay**. Variations of this technique can be used to identify components of complexes containing the protein of interest. These complexes can be other proteins (co-IP), chromatin (chromatin-IP; ChIP), or even ribonucleoproteins (RIP). For example, if a scientist is interested in whether a certain protein, called X, interacts with another protein, called Y, in a bacterial or eukaryotic cell, one approach is to mix the cell extracts with antibodies to protein X. Then, agarose beads coated with protein A are added. The protein A traps the Fc portion of the antibodies bound to protein X and any other protein bound to protein X, including protein Y. The beads are allowed to settle or are briefly centrifuged at low speed and are washed to remove all unbound protein. If protein Y is bound to protein X, it will be trapped on the beads along with protein X. The bound proteins can be eluted from the beads by harsher conditions (such as lower pH). The fractions containing protein X, along with protein Y, are separated by gel electrophoresis, and the identity of protein Y is determined by mass spectrometry (see chapter 8 for more details).

To make matters worse, these antibiotic resistances are often carried on mobile genetic elements. For example, resistance to β-lactam antibiotics such as methicillin, from which MRSA gets its name, is carried on a mobile genetic element called the **staphylococcal cassette chromosome, *mec* (SCC*mec*)**. The SCC*mec* carries the gene *mecA*, which encodes a variant of a penicillin-binding protein (PBP2a) that still carries out its normal transpeptidation activity required for peptidoglycan crosslinking in the presence of β-lactams. Instead of the *mecA* system, some strains of *S. aureus* express a β-lactamase (BlaZ) in response to the presence of β-lactam antibiotics (see Figure 16-9).

Regulation of virulence. Many of the *S. aureus* virulence genes, especially those encoding surface adhesins and exoproteins, are regulated by quorum-sensing mechanisms that sense bacterial population density (quorum-sensing, including the Agr system that regulates many of the virulence factors in *S. aureus,* is covered in detail in chapter 14). When cells are at low density in the early stages of growth, adhesin genes are preferentially expressed. These adhesins help the bacteria adhere to surfaces inside the host where they can begin to grow. Once colonization is established and the bacteria grow to a certain density, not only do nutrients become limiting, but the host immune system starts to combat the infection. In response to these challenges, the bacteria turn off their adhesin genes and begin expressing their various toxins and exoenzymes. Several global transcription regulators couple virulence factor expression, biofilm formation, bacterial metabolism, and antibiotic resistance to host central metabolism and immune responses, which together allow the bacteria to spread within the host.

Staphylococcus epidermidis—Accidental Pathogen

S. epidermidis, as its name might suggest, is found primarily on human skin. Unlike *S. aureus,* which colonizes only a fraction of humans at any given time, virtually everyone is colonized by *S. epidermidis*. In fact, it is likely that *S. epidermidis* colonization plays a role in protecting the skin from unwanted pathogenic species, such as *S. aureus.* In contrast to coagulase-positive *S. aureus*, *S. epidermidis* is the most notable and best-studied member of the coagulase-negative *Staphylococcus* (CoNS) species. Colonies of *S. epidermidis* are a chalky white, unlike the golden colonies of *S. aureus*. These colonies are familiar to many students and microbiologists, as its prevalence on human skin makes *S. epidermidis* a common contaminant in cultures of other bacterial species.

Like *S. aureus*, *S. epidermidis* can survive for long periods of time in the external environment. This hardiness, along with its ubiquity and ability to form robust biofilms (Figure 18-6), has made *S. epidermidis* a major problem for implanted abiotic medical devices, such as indwelling catheters, pacemakers, or joint prostheses. *S. epidermidis* readily binds to plastic implants, where it forms biofilms that extend into tissue. These biofilms can then shed bacteria into the bloodstream, leading to sepsis. Although *S. epidermidis* can enter the body through contaminated implanted devices, it can also be directly introduced into the human body from the contaminated skin of doctors if there are small tears in their surgical gloves. Once the bacteria have established a

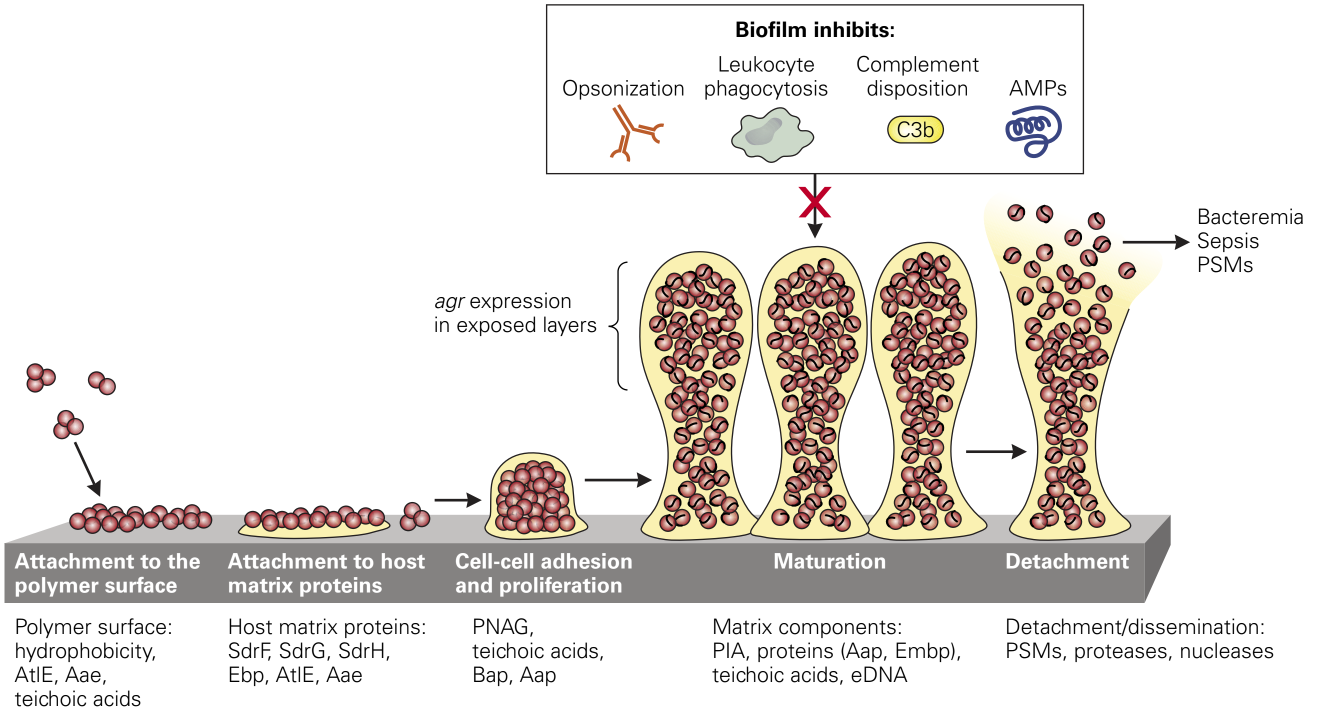

Biofilm inhibits:
Opsonization
Leukocyte phagocytosis
Complement disposition
C3b
AMPs
Bacteremia
Sepsis
PSMs
agr expression in exposed layers
Attachment to the polymer surface
Attachment to host matrix proteins
Cell-cell adhesion and proliferation
Maturation
Detachment
Polymer surface: hydrophobicity, AtlE, Aae, teichoic acids
Host matrix proteins: SdrF, SdrG, SdrH, Ebp, AtlE, Aae
PNAG, teichoic acids, Bap, Aap
Matrix components: PIA, proteins (Aap, Embp), teichoic acids, eDNA
Detachment/dissemination: PSMs, proteases, nucleases

Figure 18-6. Steps in biofilm formation and effects of biofilms on immune evasion in *Staphylococcus epidermidis*. Shown are the steps involved in staphylococcal biofilm development: attachment to an abiotic surface or to host matrix proteins, proliferation, production of cell-cell adhesive matrix components and maturation of the biofilm, and finally detachment and dispersal of the cells, which can lead to bacteremia and sepsis. PSMs, phenol-soluble modulins (surfactant-like molecules);PIA, polysaccharide intercellular adhesion exopolysaccharide; Aap, accumulation-associated protein; Embp, extracellular matrix binding protein; eDNA, extracellular DNA. Adapted from Otto M. 2018. *Microbiol Spectr* **6:**GPP3-0023-2018.

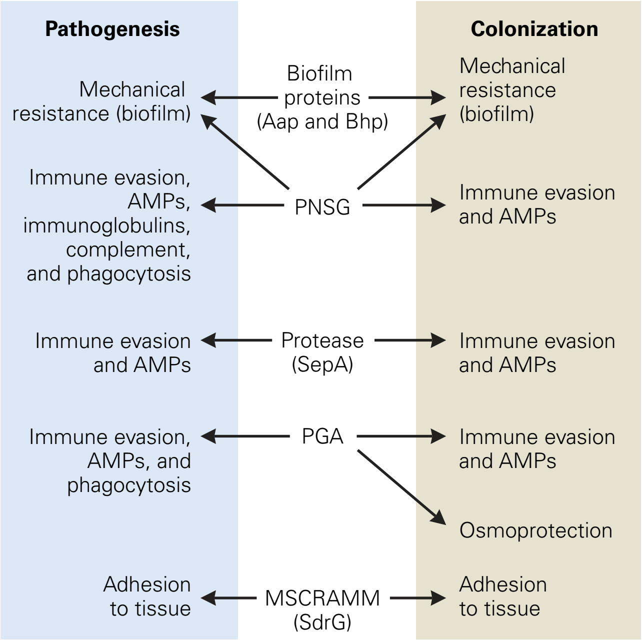


Figure 18-7.Factors that contribute to colonization and pathogenesis of *S. epidermidis*. In animal models, the roles of poly-N-acetylglucosamine (PNAG), poly-γ-glutamic acid (PGA) capsule, and the MSCRAMM SdrG in infection have been determined. Other roles are based on *in vitro* experiments and environmental challenges during colonization and infection. Adapted from Otto M. 2009. *Nat Rev Microbiol* **7:**555–567, with permission.

biofilm, it is difficult to eliminate them using antibiotics, as sessile bacteria in biofilms are naturally resistant to antibiotics, not to mention the prevalence of various antibiotic resistance genes. Moreover, biofilms provide considerable protection against several different elements of the innate immune system (Figure 18-6). In the case of contaminated implants, it is often necessary to surgically remove the implant, then treat the patient with a hefty course of antibiotics before finally performing another operation to introduce a new, hopefully sterile, implant.

Efforts have been made to prevent infections by plastic-adhering bacteria, such as *S. epidermidis,* by impregnating catheters and other implants with antibiotics or other bactericidal compounds like gallium, copper, zinc, or colloidal silver. While this approach does appear to help by preventing biofilm formation, it is not a panacea. Prevention of contamination in the first place through good hygienic practices, such as handwashing, proper glove wearing and careful scrutiny of medical implants, is key to combating infection with *S. epidermidis*.

Virulence factors. Unlike *S. aureus*, which carries a wide assortment of aggressive virulence factors and toxins, *S. epidermidis* is significantly more mild-mannered. It carries only a single class of inflammation-causing peptide that can pass as weak toxins, **phenol-soluble modulins** (**PSMs**). There is no seemingly endless list of superantigens and other nasty agents that cause severe harm to the human host. Rather, the vast majority of factors that enable *S. epidermidis* to cause disease are precisely those that enable it to colonize the skin (Figure 18-7). These factors promote adherence, confer resistance to mechanical disruption, confer tolerance of high salt or osmotic pressure, and help fend off the innate immune defenses of the skin, such as antimicrobial peptides (AMPs) and skin-associated-lymphoid tissue (SALT) (see chapter 2).

S. epidermidis employs several different mechanisms for protection against changes in osmolarity. It carries eight sodium ion/proton pumps and six distinct transport systems that take up different osmoprotectants. Additionally, *S. epidermidis* also produces a capsule-like exopolymer consisting of **poly-γ-glutamic acid (PGA)** under high salt conditions that further protects from osmotic stresses. PGA also contributes to biofilm formation and protects the bacteria against AMPs and phagocytosis by neutrophils. *S. epidermidis* biofilms are mixtures of adhesive proteins, exopolysaccharides (PGA), and released nucleic acids. Formation of these biofilms involves the participation of numerous MSCRAMMs linked by sortase to the *S. epidermidis* surface (Figure 18-6).

Biofilm formation by *S. epidermidis* goes through a series of stages (Figure 18-8A). The first occurs when individual cells attach to surfaces through hydrophobic interactions between the plastic surface and bacterial surface proteins. Cell wall **teichoic acids (TAs)** attached to the peptidoglycan may also interact with plastic surfaces. Biofilm formation in tissue involves MSCRAMMs that bind to collagen, fibrinogen, and other extracellular matrix proteins. Next, intercellular aggregation begins to occur, mediated by exopolysaccharide adhesions, such as poly-*N*-acetylglucosamine (PNAG), and various biofilm-associated adhesion proteins. Like PGA, PNAG protects *S. epidermidis* from AMPs and phagocytosis. Interestingly, PNAG is also produced by *S. aureus* specifically during *in vivo* infection. PNAG has garnered a lot of attention as a promising vaccine candidate because immunization with it has been shown to be protective against a wide range of staphylococcal infections. Once the biofilm has matured into its final structure, it begins to detach and release planktonic cells. Similar to *S. aureus*, this final dissemination step is regulated by the Agr quorum-sensing system (see Figure 14-19). Agr activation leads to the production of PSMs and proteases that facilitate bacterial spread inside the host.

An important part of *S. epidermidis* colonization is its ability to destroy AMPs produced by the skin (Figure 18-6). *S. epidermidis* uses the two-component system ApsS/R to respond to the presence of AMPs (Figure 18-8). Two-component regulatory systems were covered in depth in chapter 14. Cationic AMPs are sensed by sensor histidine kinase ApsS, which phosphorylates its cognate response

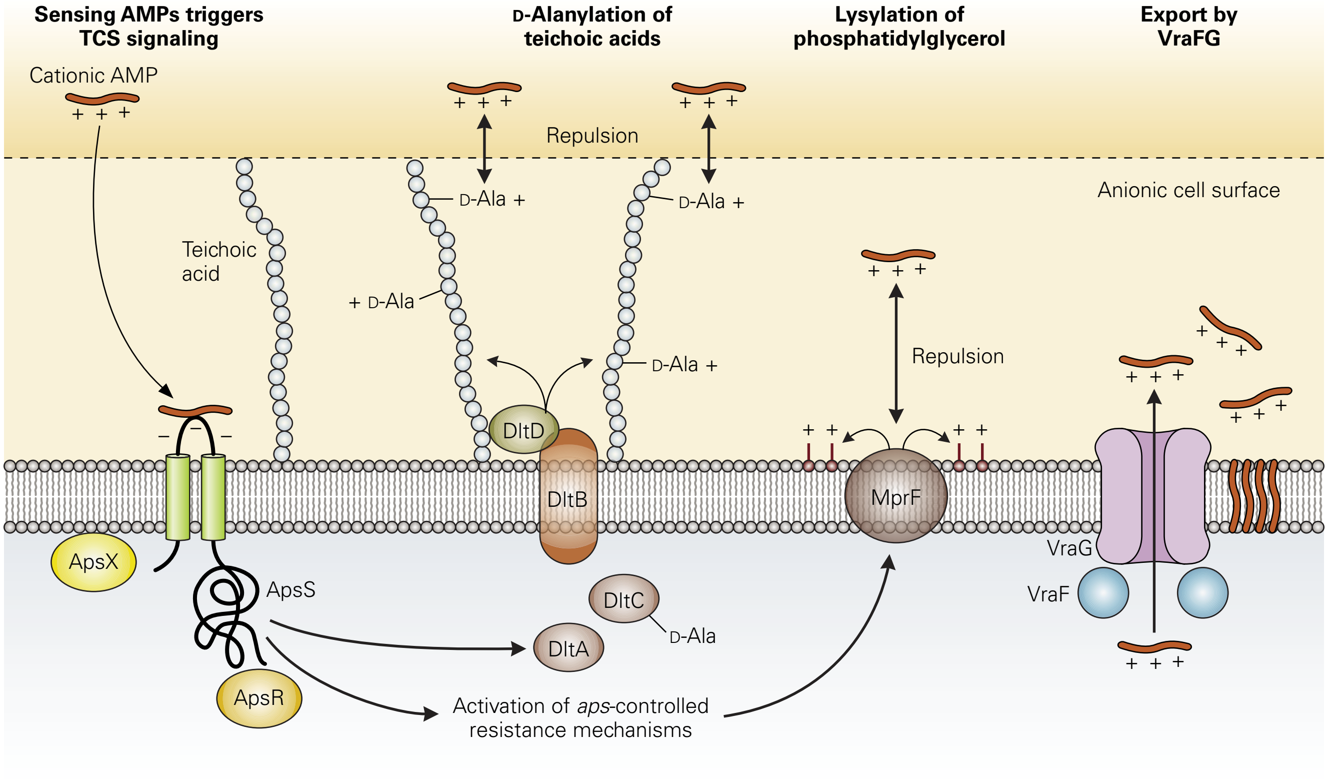


Figure 18-8. The antimicrobial peptide sensor Aps regulatory system in *S. epidermidis*. Cationic antimicrobial peptides (AMPs) attach to the negatively charged bacterial surface and membrane by electrostatic interactions, a prerequisite for AMP antimicrobial activity that is often based on pore formation in the bacterial cytoplasmic membrane. The *Staphylococcus epidermidis* ApsS AMP sensor has one short extracellular loop with a high density of negatively charged amino acid residues that interact with cationic AMPs. Transduction of this interaction signal through ApsS and the essential accessory protein ApsX, which has an unknown function, triggers response regulator ApsR-mediated expression of key AMP resistance mechanisms. D-Alanylation of teichoic acids is carried out by the products of the *dlt* operon. Lysylation of phosphatidylglycerol is catalyzed by the MprF enzyme. Both surface modifications result in a decreased negative charge of the cell surface and membrane, respectively, leading to repulsion of cationic AMPs. The VraF and VraG ABC transporter also promotes resistance to AMPs by functioning as an AMP exporter. Adapted from Otto M. 2009. *Nat Rev Microbiol* **7**:555-567, with permission.

regulator AspR. Phosphorylated AspR turns on genes that encode proteins that add D-alanine groups to cell wall TAs and lysine groups to phospholipids in the cell membrane. These modifications increase the positive charge of the cell surface, which repels cationic AMPs, preventing them from reaching the bacterial cell membrane. *S. epidermidis* also produces efflux pumps that actively remove AMPs from the cytoplasmic membrane, and several secreted proteases that likely contribute to degrading AMPs.

Comparisons between S. epidermidis and S. aureus. *S. aureus* and *S. epidermidis* share a number of different systems, but they also have key differences that greatly impact their respective disease manifestations. Both species use the Agr quorum-sensing system and produce species-specific autoinducing peptides (AIPs) (see Figures 14-22 and 14-23). While induction of the Agr system in *S. aureus* leads to production of a full range of toxins, induction of the same system in *S. epidermidis* leads to production of PSMs but no powerful toxins. Notably, the *S. epidermidis* AIPs inhibit the Agr system of most subgroups of *S. aureus*, whereas the AIP from only one rare subgroup of *S. aureus* inhibits the Agr system of *S. epidermidis*. Thus, *S. epidermidis* seems to win the interference battle against *S. aureus*. It has been speculated that this property may contribute to *S. epidermidis* predominating in skin colonization over *S. aureus*. In fact, *S. epidermidis* may play such an important role in keeping *S. aureus* in check that its elimination from the skin might actually cause microbiota shift diseases.

Another interesting comparison between these two species concerns the prevalence of SCC*mec* cassettes in *S. epidermidis* compared to *S. aureus*. Nearly every clinical isolate of *S. epidermidis* isolated in the United States and some other countries is methicillin resistant and carries the SCC*mec* cassette. Furthermore, methicillin-resistant *S. epidermidis* isolates almost always carry plasmids or other elements imparting resistance to many other antibiotics, including tetracyclines, aminoglycosides, and macrolides. As a result, methicillin-resistant *S. epidermidis* is actually more endemic in hospitals than MRSA. At this stage, only vancomycin can be used with reasonable success to treat catheter infections with *S. epidermidis*, but unfortunately strains with intermediate resistance to vancomycin have begun to appear.

It has been suggested that transfer of SCC*mec* from an *S. epidermidis* contributed to the emergence of CA-MRSA strains. Interestingly, horizontal movement of mobile genetic elements might actually be unidirectional from *S. epidermidis* to *S. aureus*, which would explain why *S. epidermidis* has not picked up toxin genes from *S. aureus*. The basis for this unidirectional transfer may be a molecular defense system (called **CRISPR-Cas**), which is present in *S. epidermidis* but not *S. aureus* isolates. CRISPR, which stands for "clustered regularly interspaced short palindromic repeats," and CRISPR-associated (Cas) proteins function in many bacteria as a quasi-immune system to confer resistance to exogenously introduced genetic elements, such as plasmids or bacterial phages, by targeting them for degradation (for detailed discussion of CRISPR-Cas systems, see chapter 7).

Streptococcus pneumoniae—"Captain of All the Men of Death"

Over a century ago, *Pneumococcus* was coined the new "Captain of the men of death" by Sir William Osler (1849–1919), who observed that the death rate from pneumonia during his time had exceeded that of "consumption" (tuberculosis, the previous "Captain"). The Gram-positive bacterium *S. pneumoniae* (also referred to as pneumococcus) is arguably the most common cause of infectious disease deaths in the world. Annually, nearly 1.2 million deaths worldwide from lower respiratory infections are caused by *S. pneumoniae*, approximately 70% of which are in children under the age of 5 or in elderly people over the age of 70. In addition to age, other major risk factors for pneumococcal disease include debilitation due to poverty, alcoholism, untreated chronic diseases like diabetes, immunosuppression from immune system defects, cancer therapy, or infections (e.g., with HIV), and antecedent respiratory viral infections, especially by influenza. In fact, more than half of the millions of fatalities during the worldwide influenza pandemic of 1918 stemmed from *S. pneumoniae* infection.

Risk of invasive disease also depends on the strain of *S. pneumoniae*. There are at least 98 different subgroups of *S. pneumoniae* strains, defined by the composition of their exopolysaccharide capsules (i.e., serotype). However, these groups differ from each other in other ways as well. Genomic comparisons of different strains have shown that their genomes can differ by as much as 10%, with a significant portion of the observed diversity concentrated in pathogenicity islands that determine the type and severity of disease they cause. Since *S. pneumoniae* is naturally competent, there is considerable genome plasticity among *S. pneumoniae* subgroups, and even between related species such as the oral bacterium *Streptococcus mitis*.

The deadliest forms of *S. pneumoniae* disease are caused by invasive infections resulting in pneumonia, bacteremia, and meningitis, but *S. pneumoniae* is also a common cause of the less serious childhood disease, otitis media (earache). *S. pneumoniae* colonizes the nasopharynx, which is thought to be a prerequisite for the more invasive diseases (Figure 18-9A). Recent advances in modeling pneumococcal transmission and colonization in mouse pups support this idea. With the possible exception of pneumonia, pneumococcal diseases do not typically lead to transmission among hosts. Additionally, pneumococcal pneumonia is not considered contagious enough in the hospital setting to require isolation. However, community outbreaks of pneumonia have been reported that are consistent with transmission through coughing.

In most cases, colonization is asymptomatic, or at worst results in a mild runny nose, the duration of which strongly depends on the serotype subgroup, but usually lasts for weeks in adults and for months in children. This asymptomatic colonization is the major reservoir for transmission of *S. pneumoniae* among humans. Interestingly, most people are usually colonized by only one serotype of *S. pneumoniae* at a time. This intraspecies competition is partly mediated by the production of serotype-specific antibacterial peptides called **pneumocins**. Strains that secrete these pneumocins also produce cognate immunity proteins, which protect against self-killing.

In addition to its epidemiological impact, *S. pneumoniae* has served as an important bacterial model system used to study fundamental molecular processes. In 1923, Oswald Avery (1877–1955), along with Michael Heidelberger (1888–1991) and other colleagues, found that the *S. pneumoniae* capsule was an immunogenic exopolysaccharide. This discovery helped launch the field of immunochemistry. In 1928, Frederick Griffith (1879–1941) demonstrated that an avirulent strain of pneumococcus could be transformed into a lethal form by co-inoculating a mouse with a mixture of live avirulent bacteria and dead heat-killed virulent bacteria (see Figure 7-8). At the time, this transforming factor was believed to be a protein; however, in 1944, Avery, along with Colin MacLeod (1909–1973) and Maclyn McCarty (1911–2005), demonstrated that this transforming factor was, in fact, DNA. The work by Griffith, Avery, and others would lay the foundation for the field of molecular genetics.

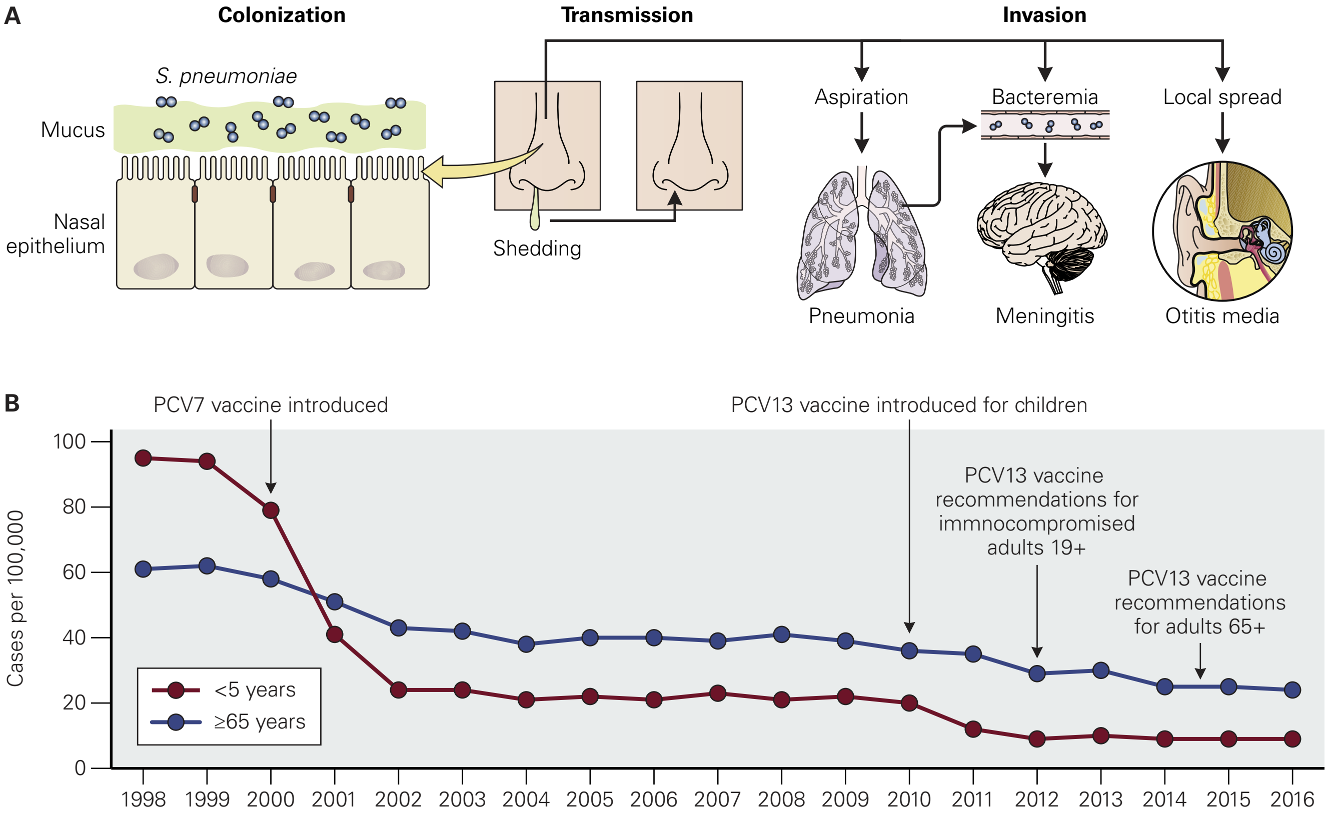


Figure 18-9. ***Streptococcus pneumoniae*** **infections and control by pneumococcal vaccination. (A)** Transmission, colonization, and invasive diseases caused by *Streptococcus pneumoniae*. Carriage by colonization is required for transmission to other individuals and for invasive diseases in carriers. The bacteria are shed and transmitted through nasal secretions. Bacteria spread beyond the mucosa of the upper respiratory tract leads to invasive diseases. Adapted from Weiser JN, Ferreira DM, Paton JC. 2018. *Nat Rev Microbiol* **16:**355–367. **(B)** Incidence of pneumococcal disease in children (red) and older adults (blue) in the United States before and after introduction of the PCV-7 vaccine and later the PCV-13 vaccine. Data from Centers for Disease Control and Prevention (https://www.cdc.gov/abcs/reports-findings/survreports/spneu-types.html).

Vaccines. When *S. pneumoniae* reaches the lungs, it first encounters alveolar macrophages, then as infection progresses, neutrophils also enter the area (see chapter 3). The main defense of *S. pneumoniae* against these phagocytic cells is its exopolysaccharide capsule. The oldest and most widely used method for prevention of pneumococcal pneumonia is a vaccine that consists of antigens from the 23 most common capsular types. This 23-valent capsular vaccine has proven to be very safe and is routinely given to the elderly, who are at greatest risk of contracting pneumonia. Unfortunately, its efficacy, especially among the elderly, is only around 60%. Furthermore, the 23-valent pneumococcal vaccine is useless in infants, who lack T-cell-independent responses to generate antibodies to long-chain polysaccharides.

Given the fact that influenza is frequently a precursor to secondary *S. pneumoniae* infections, another preventive strategy has been to require health care workers that care for the elderly to have anti-influenza vaccinations. This indirect immunization builds a shield of protection (so-called "**herd immunity**") around the elderly residents. During 2009, the issue of requiring influenza vaccination of health care workers resurfaced in connection with the swine flu (H1N1) pandemic, where many health care workers refused the vaccination. During the pandemic, over half of the fatalities were due to secondary pneumococcal infection.

Since capsule vaccines are not effective in young children, other forms of immunization are required. As we discussed in chapter 17, covalently linking carbohydrates to peptide antigens can induce MHCII-mediated T-cell-dependent responses to the carbohydrates, even in infants. In 2000, such a protein conjugated-capsule vaccine, **PCV-7 (pneumococcal conjugated 7-valent vaccine)**, was introduced. The capsule types covered by PCV-7 were the seven most prevalent serotypes recovered in clinical isolates from developed countries at the time. This vaccine has been highly successful in the United States and other developed countries (Figure 18-9B) with invasive pneumococcal diseases dropping by nearly 80% for infants under 2 years of age. The vaccine reduces the rates of pneumococcal carriage and transmission by immunized children, thereby contributing to herd immunity that indirectly protects unvaccinated infants in crowded day care settings.

After its introduction, most infants in the United States routinely received PCV-7, making it a "blockbuster" product for the pharmaceutical company that produced it. Unfortunately, the serotypes that cause invasive pneumococcal diseases in developed countries only partly overlap those in developing countries. Furthermore, conjugated vaccines are often too expensive for use in the developing world. Alarmingly, since the PCV-7 vaccine was released, new strains have emerged as major causes of invasive pneumococcal disease in infants. In an effort to expand the coverage of the conjugate vaccine, a new 13-valent conjugated vaccine, PCV-13, was introduced in 2010. Attempts have also been made to vaccinate young children in developing countries with PCVs against serotype strains clinically relevant to those countries. Meanwhile, a search continues for pneumococcal surface proteins that can serve as vaccine candidates for eliciting a protective immune response. One challenge has been that many pneumococcal surface proteins exhibit variability among different serotype strains. Recently, two highly conserved surface proteins involved in peptidoglycan remodeling (PcsB) and in signaling during cell division (serine-threonine kinase StkP) were identified as strong antigens in humans and are being explored as potential vaccine candidates.

Colonization and virulence factors. As was mentioned earlier in the chapter, *S. mitis* is an oral commensal closely related to *S. pneumoniae*. Genomic analyses have suggested that *S. pneumoniae* may have evolved from an ancient *S. mitis* progenitor. Consistent with this notion, the *S. pneumoniae* genome contains a large number of truncated genes that are still intact in *S. mitis*. Loss of these genes may contribute to the ability of *S. pneumoniae* to colonize the nasopharynx and other niches during invasive infections. Conversely, *S. pneumoniae* has acquired key virulence genes absent in *S. mitis*, including numerous sugar transporters, capsule biosynthetic enzymes, the pore-forming toxin **pneumolysin**, and **hyaluronate lyase**, an enzyme that degrades hyaluronic acid polysaccharide in the host extracellular matrix (Figure 18-10).

Colonization of the nasopharynx by *S. pneumoniae* starts with adherence to nasal surfaces. Isolates that effectively colonize seem to express less capsule than those isolated during invasive disease. These isolates can be differentiated by their growth on some media with the former forming transparent colonies and the latter forming opaque colonies. The switch between these two phenotypes is a form of phase variation mediated by inverted repeat sequences recognized by a restriction-modification system. In general, pneumococcal cells that produce less capsule adhere significantly better to eukaryotic cells in culture and produce certain forms of biofilms more effectively. Nevertheless, capsule formation does seem to play a role in preventing entrapment in nasal mucus and allowing nonmotile *S. pneumoniae* cells to reach the epithelial cells lining the nasopharynx.

S. pneumoniae employs several different mechanisms to adhere to the apical surface of epithelial cells. Some strains produce pili that are covalently attached to peptidoglycan by a specialized sortase (Figure 18-4A). These pili carry adhesins that bind to specific receptors on host cell surfaces. Because pili are large extracellular appendages, they are likely involved in initiating contact with host cells. Once contact is made with the host cell, *S. pneumoniae* partially sheds its extracellular capsule exposing additional adhesins, including the lipoprotein **pneumococcal surface adhesin** (**PsaA**), which binds to the epithelial cadherin, **pneumococcal adherence and virulence factor A** (**PavA**), which binds to fibronectin, NADH oxidase (Nox), which binds to extracellular matrix component laminin alpha-5, and the lectin fructose bisphosphate aldolase (FBA), which binds to the flamingo cadherin receptor. It is worth noting that in addition to being adhesins, these proteins have enzymatic activities independent of their roles as adhesion molecules.

S. pneumoniae also secretes roughly 15 different **choline-binding proteins (CBPs)**. These CBPs bind to the **phosphorylcholine (P-Cho)** groups that are incorporated into the cell wall TAs and LTAs, where they also mediate adherence. Interestingly, there are also host proteins that function in a similar way. **Platelet-activating factor receptors (PAFR)** on the surfaces of epithelial cells also bind to the P-Cho groups of wall TAs and LTAs. This binding is a form of molecular mimicry as platelet-activating factor (PAF), the normal ligand for PAFR, contains a P-Cho group. This molecular mimicry has an additional consequence in that *S. pneumoniae* binding to PAFR not only promotes adherence, but it also activates the receptor, setting off a signaling cascade in the host cells that triggers innate responses.

Aside from protein adhesins, *S. pneumoniae* binds to carbohydrate groups attached to the glycosphingolipids in epithelial cell membranes. *S. pneumoniae* encodes several exoglycosidases that remove sugar residues from host glycoproteins. These exoglycosidases are covalently linked to the peptidoglycan by sortases. In addition to providing the bacteria with a source of food, these hydrolytic enzymes, such as the **neuraminidase NanA**, which cleaves off sialic acid groups, expose new sites on the host cell surface for *S. pneumoniae* to bind. In the case of NanA, the released sialic acid also acts as a signal for certain two-component regulatory systems, which upregulate factors that promote biofilm formation and progression to invasive disease.

S. pneumoniae is primarily an extracellular pathogen that causes disease by inducing severe inflammatory responses. However, *S. pneumoniae* also has ways of breaching epithelial

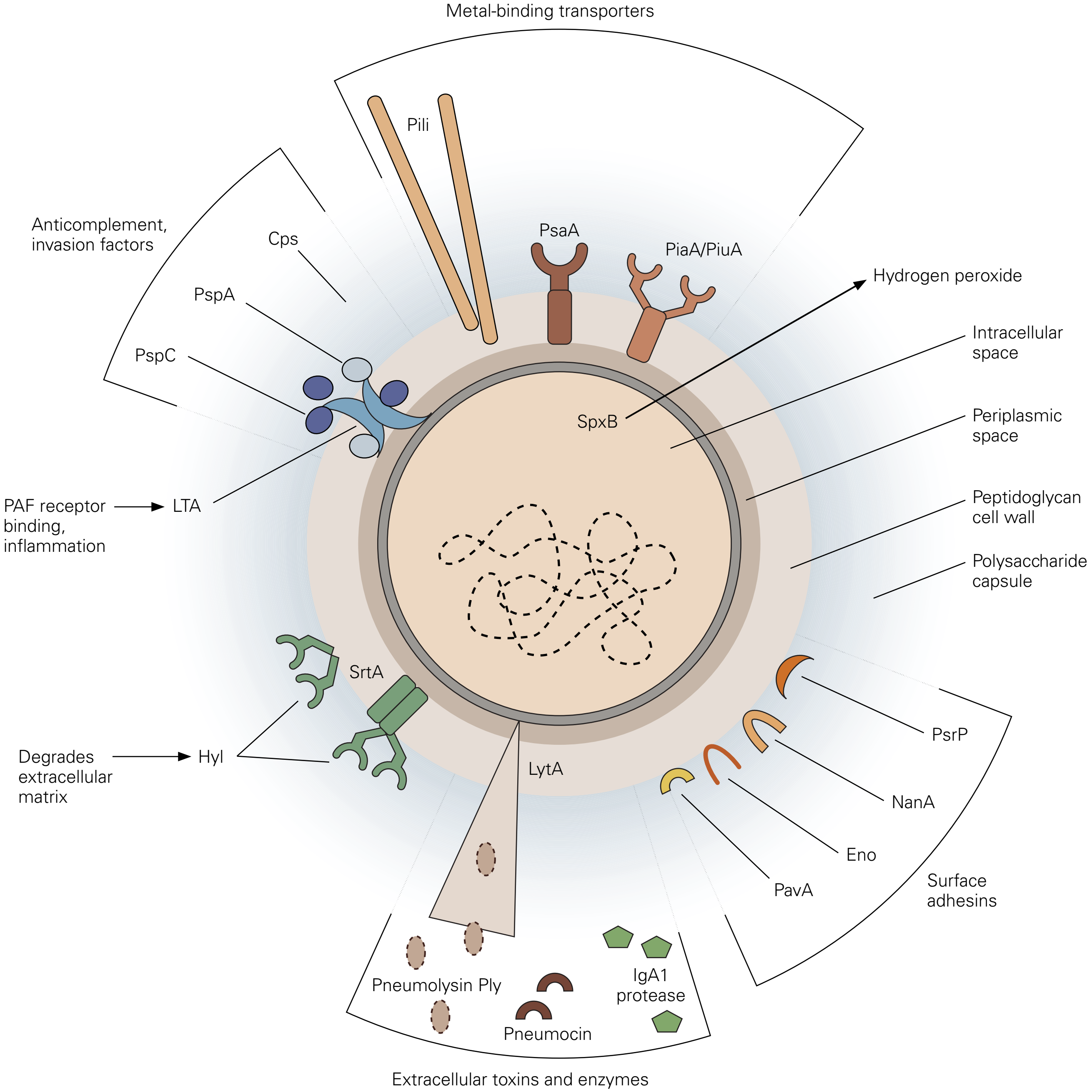

Metal-binding transporters
Pili
PsaA
PiaA/PiuA
Hydrogen peroxide
Anticomplement, invasion factors
Cps
PspA
PspC
Intracellular space
SpxB
Periplasmic space
PAF receptor binding, inflammation
LTA
Peptidoglycan cell wall
Polysaccharide capsule
SrtA
PsrP
Degrades extracellular matrix
Hyl
LytA
NanA
Eno
PavA
Surface adhesins
IgA1 protease
Pneumolysin Ply
Pneumocin
Extracellular toxins and enzymes

Figure 18-10. Virulence factors of *Streptococcus pneumoniae*. Shown are the known virulence factors associated with pneumococcal disease. PsaA, pneumococcal surface antigen A; PiaA/PiuA, pneumococcal iron acquisition and uptake; PsrP, pneumococcal serine-rich repeat protein; NanA, neuraminidase; Eno, enolase; PavA, pneumococcal adhesin and virulence; LytA, autolysin; SrtA, general sortase; Hyl, hyaluronate lyase; PspC, pneumococcal surface protein C; PspA, pneumococcal surface protein A; Cps, polysaccharide capsule; SpxB, pyruvate oxidase, Ply, pneumolysin. Adapted from Van der Poll T, Opal. 2009. *Lancet* **374:**1543–1556, with permission.

cell layers to reach underlying tissues and the bloodstream. In chapter 4, we introduced **transcytosis** in the context of secretory IgA (sIgA). Transcytosis is a process where large macromolecules are taken up into cells through endocytosis, transported in vesicles through the cell, and deposited on the opposite side of the cell. Normally, sIgA binds the **poly-Ig receptor** on the basal surface and is transported to the apical surface. *S. pneumoniae* has evolved a way to hijack this system to penetrate epithelial tissue by binding to the poly-Ig receptor when it is exposed on the apical side of the cell, where it can then be transcytosed through the cell to the basal side of the epithelial layer. This binding is mediated through CbpA (also called PspC), one of the CBPs produced by *S. pneumoniae*. Another more straightforward way for *S. pneumoniae* to breach epithelial cell layers is to slip through natural openings used by neutrophils to enter the nasal cavity. This process is called **occult bacteremia** (i.e., the presence of bacteria in the bloodstream of febrile patients with no apparent site of infection).

Within three days of colonization, the host strikes back with an influx of neutrophils to the lumen of the nasal cavity. *S. pneumoniae* resists this onslaught from the host through a couple different mechanisms. These include releasing a protease that specifically cleaves human sIgA, decorating the surface LTA with positively charged D-alanine residues to ward off host cationic AMPs (see Figure 18-8), and deacetylating the N-acetylglucosamine residues in its peptidoglycan to protect it from host lysozyme cleavage. Additionally, the capsule inhibits opsonization by sterically blocking the binding of complement factors and IgG molecules to receptors on neutrophils and macrophages. The capsule also reduces deposition of complement factors on the bacterial surface and the capture of pneumococcal cells in neutrophil extracellular traps (NETs; see chapter 3). Similarly, *S. pneumoniae* CBPs also prevent activation of the alternative complement cascade. CbpA binding to the poly-Ig receptor, which we mentioned in the context of transcytosis, and its binding to factor H inhibits formation of C3b. PspA, another CBP, protrudes through the capsule and blocks the binding of complement component C3b to factor B. PspA also binds host lactoferrin, which further protects the bacterial cells.

Several elements of the host defenses are stimulated by interactions with pneumococcal components (called PAMPs [pathogen-associated molecular patterns]; see chapter 3) (Figure 18-11). *S. pneumoniae* cells release the soluble cytolytic toxin, pneumolysin. Interestingly, *S. pneumoniae* that do not produce pneumolysin actually persist longer in the nasopharynx than those that do. One explanation for this seemingly paradoxical observation is that bacterial induction of inflammation furthers transmission, such that it is still evolutionarily advantageous to produce the toxin despite being cleared more rapidly. Pneumolysin does not seem to be transported directly out of cells and is likely released by the activity of **cell wall hydrolases**, such as the N-acetylmuramoyl-L-alanine amidase **LytA**, which cause *S. pneumoniae* cell autolysis, particularly in stationary phase cells. Besides its cytolytic activity, pneumolysin directly binds to TLR4, inducing additional proinflammatory responses. Once released, pneumolysin assembles in the cholesterol-containing membranes of the host cells and produces large donut-shaped transmembrane pores. As the concentration of pneumolysin builds up in the membranes, the beating of cilia is inhibited, cytokine and chemokine release is further stimulated, and CD4$^+$ T cells and chemotaxis of immune cells are activated.

S. pneumoniae autolysis also releases inflammatory substances like peptidoglycan and LTA, which interact with toll-like receptor 2 (TLR2) of the innate immune system (see chapter 3). TLR2 stimulation leads to production of the cytokine interleukin 17 (IL-17) by CD4$^+$ T cells and induces the release of proinflammatory cytokines and chemokines. Release of IL-17 results in recruitment of neutrophils, monocytes, and macrophages to the mucosal surface to clear pneumococcus from the nasopharynx. This antibody-independent involvement of CD4$^+$ T cells in controlling pneumococcal colonization provides an explanation for why people infected with HIV, which reduces the number of CD4$^+$ T cells, are highly susceptible to persistent pneumococcal colonization and invasive disease. Eventually, the mucosal immune system starts producing sIgA antibodies directed against pneumococcal surface epitopes to further eliminate the bacteria. Another host defense molecule, called C-reactive protein (CRP), binds to P-Cho groups in the pneumococcal cell wall TAs and LTAs. This complex then binds the C1 component and actives the classical complement pathway (see chapter 3).

S. pneumoniae secretes copious amounts of hydrogen peroxide produced by the enzyme **pyruvate oxidase (SpxB)** (see Figure 18-10), which is involved in synthesizing additional ATP from pyruvate. This hydrogen peroxide stimulates cytokine production by surrounding host cells and possibly inhibits the growth of competing microbial species for colonization of the nasopharynx. Several genes protect *S. pneumoniae* from this endogenously produced hydrogen peroxide, including three different, regulated ABC transporters (Pia, Piu, and Pit) that take up iron and the PsaBCA transporter that takes up manganese. Note that the extracellular lipoprotein of this transporter, PsaA, was one of the adhesins we introduced earlier in this section that binds host epithelial cadherin. It is still unclear how *S. pneumoniae* protects its DNA from endogenous hydrogen peroxide concentrations that approximately match those produced by exogenous oxidative bursts in macrophages. Recent results suggest that the TpxD thiol peroxidase may play a critical role in this protection by limiting protein oxidative damage by sulfenylation of glycolytic, capsule, and nucleotide biosynthetic enzymes.

The same factors that play roles in colonization are important for invasive disease (Figure 18-12). Foremost among these is the capsule. Mutants that lack a capsule are avirulent in animal models of infection, even at extremely high bacterial doses. Pneumolysin is also well established as a critical factor for invasive diseases. The contribution of other factors is more graded and seems to depend on the specific strain and the infection model used. Several bacterial factors that strongly influence virulence do not affect colonization, but this may only reflect the limitation of the animal models available for studying colonization.

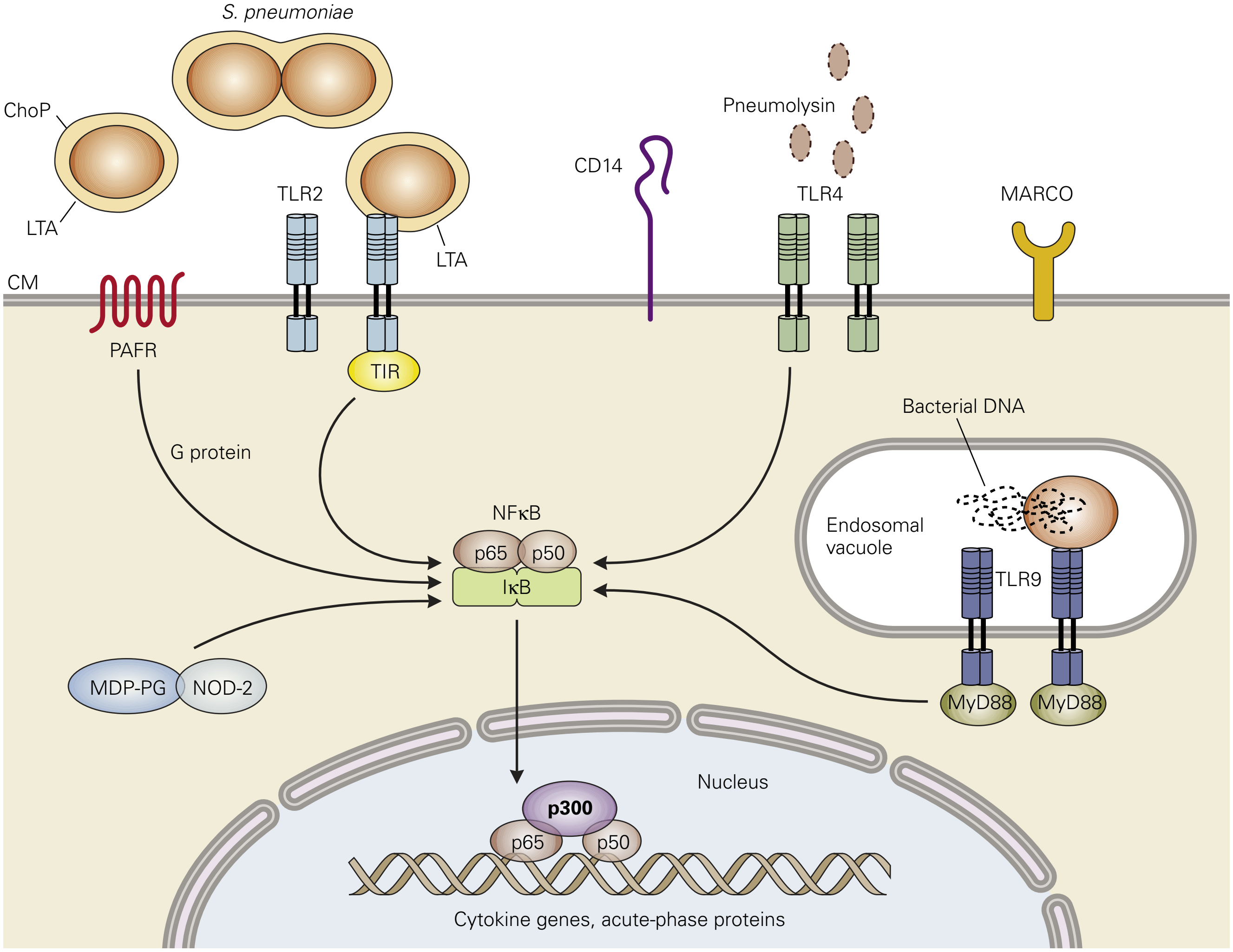


Figure 18-11. **Pattern recognition signaling receptors and pathways in pneumococcal infection.** *S. pneumoniae* is recognized as a pathogen in the lung by several TLRs, including TLR2 (with pneumococcal LTA as its major ligand), TLR4 (which recognizes pneumolysin), and TLR9 (within endosomes; interacts with bacterial DNA). Macrophage receptor with collagenous structure (MARCO) expressed by alveolar macrophages contributes to innate immune responses in lungs. PAFR is shown as a pattern recognition receptor because it recognizes pneumococcal phosphorylcholine and LTA, thereby contributing to tissue invasion. CD14 probably further helps *S. pneumoniae* invade from the airways into blood. Within cytoplasm, the muramyl dipeptide component of pneumococcal peptidoglycan (MDP-PG) is recognized by nucleotide-binding oligomerization domain (NOD-2) and can activate host defense and inflammation. ChoP, phosphorylcholine; PAFR, platelet-activating factor receptor; TIR, Toll-IL-1 receptor domain; MyD88, myeloid differentiation primary response protein-88; NFκB, nuclear factor κB; IκB, inhibitor of κB. Adapted from van der Poll T, Opal SM. 2009. *Lancet* **374:**1543–1556, with permission.

Stimulation of the host innate immune system leads to a substantial increase in the production of proinflammatory cytokines and chemokines. Macrophages use several additional signaling pathways of the innate immune system besides the ones mentioned already. The scavenger receptor MARCO expressed on the surface of alveolar macrophages and binds to *S. pneumoniae* and promotes their opsonization (Figure 18-12). Mice deficient in MARCO are highly susceptible to fatal pneumococcal infections. Pneumococcal cells internalized and degraded in phagolysosomes release their chromosomal DNA, which sets off innate immune responses mediated by TLR9. Fragments of digested pneumococcal peptidoglycan that are released into the cytoplasm through the action of pneumolysin further stimulate the inflammatory response via the NOD2 (nucleotide-binding oligomerization domain) receptor (see chapter 3). Other surface proteins, such as C-type (SIGNR1) lectins, on macrophages in the spleen also combat pneumococcal infection. These lectins mediate the uptake of capsule and *S. pneumoniae* cells and the presentation of capsule antigens to B cells.

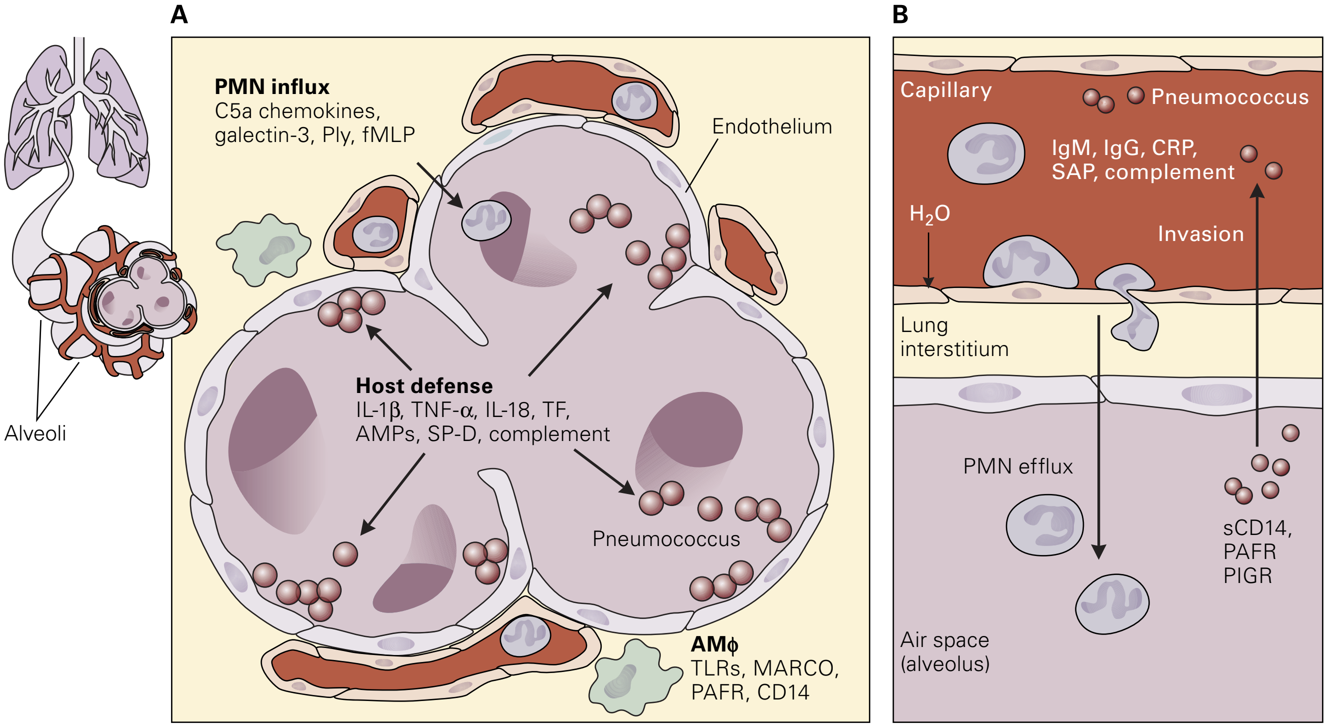


Figure 18-12. Steps in invasive *Streptococcus pneumoniae* infection. (A) Pneumococci that enter lower airways are recognized by pattern-recognition receptors, including toll-like receptors (TLRs; on epithelial cells and alveolar macrophages) and macrophage receptors with collagenous structure (MARCO; on alveolar macrophages). At low infectious doses, epithelial cells and alveolar macrophages can clear *S. pneumoniae* without help from recruited neutrophils, in part by releasing protective inflammatory mediators such as IL-1, TNF-α, IL-18, complement proteins (C), surfactant protein-D (SP-D), and AMPs. These mediators continue to have a role after infection with a high infectious dose, whereby PMNs are recruited by chemoattraction through various mediators, including C5a, galectin-3, and pneumococcal products, such as pneumolysin (Ply) and formyl-methionine-leucine-phenylalanine (fMLP). **(B)** If alveolar defense mechanisms are overwhelmed by the multiplication of pneumococci, invasion of *S. pneumoniae* into the bloodstream takes place, helped by platelet-activating factor receptor (PAFR), poly-Ig receptor (PIGR), and soluble CD14 (sCD14). In the bloodstream, several host proteins contribute to host defense, including IgM antibodies, complement proteins (C), C reactive protein (CRP), and serum amyloid peptide (SAP). TF, tissue factor; AMφ, alveolar macrophage. Adapted from van der Poll T, Opal SM. 2009. *Lancet* **374:**1543–1556, with permission.

As alveolar macrophages become overwhelmed, signals are released to recruit an influx of neutrophils. These signals include C5a complement fragment, α-chemokines, a soluble adhesion called galectin, and pneumolysin (see Figure 18-12). Some *S. pneumoniae* cells may escape to the bloodstream by slipping out of openings that allow neutrophil influx. Escape of *S. pneumoniae* from the blood is facilitated by binding to PAFR and a soluble recognition protein called cluster of differentiation 14 (CD14), which acts as a coreceptor, along with TLR2, for bacterial cell wall components. Some of these mechanisms contribute to the susceptibility of influenza patients to secondary pneumococcal pneumonia. Viral infection temporarily destroys the ciliated cells that guard the airway. Viral infections also increase the production of PAFR on lung and endothelial cells, which in turn increases that adherence of pneumococci to these cell types. Influenza impairs some of the signaling pathways mediated by the TLRs, such as TLR9, that are important for phagocytosis capacity. Cytokines produced in response to influenza also downregulate MARCO scavenger receptors on alveolar macrophages.

Many of the symptoms of pneumococcal pneumonia, such as fever and lung damage, can be accounted for by the intense and largely unrestrained inflammatory response caused by bacteria growing in the lung. Damage to endothelial cells allows blood to enter the lung and produces a common symptom of pneumonia, bloody sputum. Peptidoglycan fragments activate the alternative complement pathway and elicit IL-1 production by macrophages. LTA from the bacterial cell wall is an effective activator of the alternative complement pathway, which in turn elicits cytokine production. Most people produce antibodies to bacterial cell wall antigens, but not to capsular antigens, possibly because the capsular antigens are less antigenic. Antibodies to cell wall antigens can diffuse through the porous matrix of the capsule to the bacterial surface, where they bind and activate complement by the classical pathway. Binding of complement proteins to the cell surface contributes to more production of C5a, the chemoattractant of neutrophil chemotactic factor, but does not aid phagocytic uptake of bacteria, because C3b bound to the cell wall cannot interact with phagocyte receptors due to physical interference by the capsule. The net result is a continually expanding inflammatory response that causes considerable tissue damage but does not clear the bacteria. The fluid that accumulates in the lungs as pneumonia develops is due to leakage from blood vessels as neutrophils move through vessel walls and from activated complement components that increase vascular permeability. Damage to the area also disrupts gas exchange so that the patient literally suffocates. Ever-decreasing amounts of oxygen do not inhibit pneumococcal growth because *S. pneumoniae* is metabolically an aerotolerant anaerobe. Some of the same translocation mechanisms, such as P-Cho:PAFR and CbpA:poly-Ig receptor mentioned above, are used by *S. pneumoniae* cells to cross the blood-brain barrier and cause meningitis.

Virulence regulation. Colonization and virulence factor expression in *S. pneumoniae* does not seem to be regulated by a single master regulatory pathway, such as the Agr system in *S. aureus*. Moreover, the simplified paradigm of adhesion gene expression followed by virulence gene expression as the pathogen culture progresses from exponential to stationary phase does not apply to *S. pneumoniae*, partly because laboratory cultures begin to undergo autolysis shortly after reaching stationary phase. Instead, specific regulators and several global regulatory systems, such as catabolite repression, stringent response, and two-component systems control the genes that mediate pneumococcal colonization and virulence.

The single largest regulon (>100 genes) in *S. pneumoniae* mediates development of natural competence (see chapter 7). Competence induction is a complicated process, which partly involves accumulation of a secreted peptide that resembles a quorum-sensing autoinducer. This competence-stimulatory peptide is sensed by an autoregulatory two-component system (ComDE) that stimulates the expression of the single alternative sigma factor (ComX) in pneumococcus. RNA polymerase containing ComX stimulates transcription of late competence genes required for DNA uptake and recombination. An interesting aspect of pneumococcal competence is fratricide, where cells that become competent produce cell wall hydrolases that kill noncompetent cells in the population. Besides providing a mechanism for great genetic plasticity, induction of pneumococcal competence is a general stress response to conditions that damage DNA or disrupt cellular function. Consistent with this idea, exposure of *S. pneumoniae* to certain antibiotics that damage DNA (mitomycin), inhibit translation (kanamycin), or inhibit DNA gyrase (norfloxacin) strongly induce competence.

Antibiotic resistance. When penicillin-resistant pneumococci first appeared, some scientists assumed this was yet another example of resistance due to inactivation of the drug by a β-lactamase. However, no β-lactamase has yet been discovered in clinical isolates of *S. pneumoniae*. Instead, resistance to β-lactam antibiotics appears to be entirely due to mutations in the penicillin-binding proteins (PBPs) themselves. Interestingly, the PBPs in *S. pneumoniae* and their relatives in *S. mitis* have mosaic sequences that have arisen from the reshuffling of gene segments through recombination during transformation. One of the standard ways of overcoming β-lactam resistance in the clinic has been to treat bacterial infections with both β-lactams and a β-lactamase inhibitor (such as clavulanic acid). The β-lactamase inhibitor inactivates β-lactamase, allowing the β-lactam to kill the bacteria (see Figure 15-26). Unfortunately, because resistance to β-lactam antibiotics is completely independent of β-lactamase in the case of *S. pneumoniae*, this treatment strategy is completely ineffective.

Clinical isolates of *S. pneumoniae* are also acquiring resistance to other classes of antibiotics, such as erythromycin, tetracyclines, and chloramphenicol (see chapter 16 for mechanisms), through the spread of conjugative transposons among Gram-positive bacteria (see chapter 7). A troubling feature of these types of resistance mechanisms is that they often confer resistance to several different antibiotics within a given class. For example, erythromycin resistance imparts additional resistance to other antibiotics that act on ribosomes, such as streptogramins and lincosamides. Thus, multidrug resistance (MDR) in *S. pneumoniae* is increasing rapidly and has been recognized recently as a serious, immediate threat to human health by the CDC and WHO.

This alarming trend is probably being exacerbated by the increase in the number of adults who are at risk for persistent colonization and invasive diseases. Furthermore, because of the genetic plasticity of *S. pneumoniae*, it is highly likely that virulent strains will become increasingly resistant to antibiotics. So far, most MDR strains of *S. pneumoniae* remain sensitive to later generation quinolones, such as levofloxacin, and to vancomycin, but it is only a matter of time before their resistance cassettes find their way into *S. pneumoniae*.

Clostridium difficile—True Opportunist

The discovery of antibiotics revolutionized medicine by providing cures for many infectious diseases, which had previously caused untold suffering and death. So, it is understandable that antibiotics were viewed as miracle drugs and that physicians and the public at large would be reluctant to accept the fact that antibiotic use might have some negative aspects. That some antibiotics have toxic side effects was recognized early on, and although the connection between overuse of antibiotics and the emergence of antibiotic resistant strains was made much later, clinicians and regulatory agencies are at least beginning to address the issue. What has been even harder to accept is the idea that antibiotics being used successfully to treat one type of bacterial infection might actually cause another type of bacterial disease in the same patient. These diseases occur when antibiotics depress the resident microbiota of various body sites, thereby allowing pathogens previously kept in check by the normal resident microbiota to overgrow. **Pseudomembranous colitis,** a disease characterized by severe inflammation and ulceration of the colon, is probably the best documented example of this type of disease. Other examples are yeast vaginitis and bacterial vaginosis, two diseases that can develop in some women who take antibiotics that affect the normal vaginal microbiota and cause microbial dysbiosis (see chapter 5).

Pseudomembranous colitis was first described nearly a century ago, before the advent of antibiotics. It had been a rare disease until around 1970, when cases began to occur in hospitals, particularly among elderly patients. Because pseudomembranous colitis is often fatal and can kill within a few days, these cases caused considerable alarm. The reason for the sudden increase in pseudomembranous colitis cases turned out to be the widespread use of antibiotics, such as clindamycin, cephalosporins, and ampicillin, which inhibit the growth of the predominant genera of colonic bacteria (Figure 18-13). This gave the causative agent, *C. difficile*, which is normally present in only about 5% of the population and at only a very low abundance, the chance to overgrow in the colon environment and cause disease.

C. difficile, sometimes called "*C. diff*" in the popular press, is a Gram-positive, anaerobic, rod-shaped, motile bacterium. Like other clostridia, *C. difficile* is a spore-former and is notable for its ability to produce exotoxins. *C. difficile* causes a spectrum of diseases, called CDIs (*C. difficile* infections), which range from a mild diarrhea to pseudomembranous colitis. Now that diagnostic tests for detecting toxins in feces are available and the types of patients who are at highest risk for pseudomembranous colitis can be identified, aggressive treatment of patients who show early signs of developing full-blown disease has lessened its incidence. Also, restrictions on the use of antibiotics known to cause pseudomembranous colitis have helped to reduce the incidence of the disease even further. However, it has recently been found that *C. difficile* is widespread in the environment and can cause diseases in animals. Furthermore, CDIs caused by hypervirulent strains are no longer confined to hospitals and are increasingly being acquired in the community.

An interesting feature of pseudomembranous colitis is that many strains of *C. difficile* are susceptible to the antibiotics that precipitate episodes of disease. How then can *C. difficile* overgrow if the antibiotic is present? The reason seems to be that as antibiotic treatment comes to an end and levels

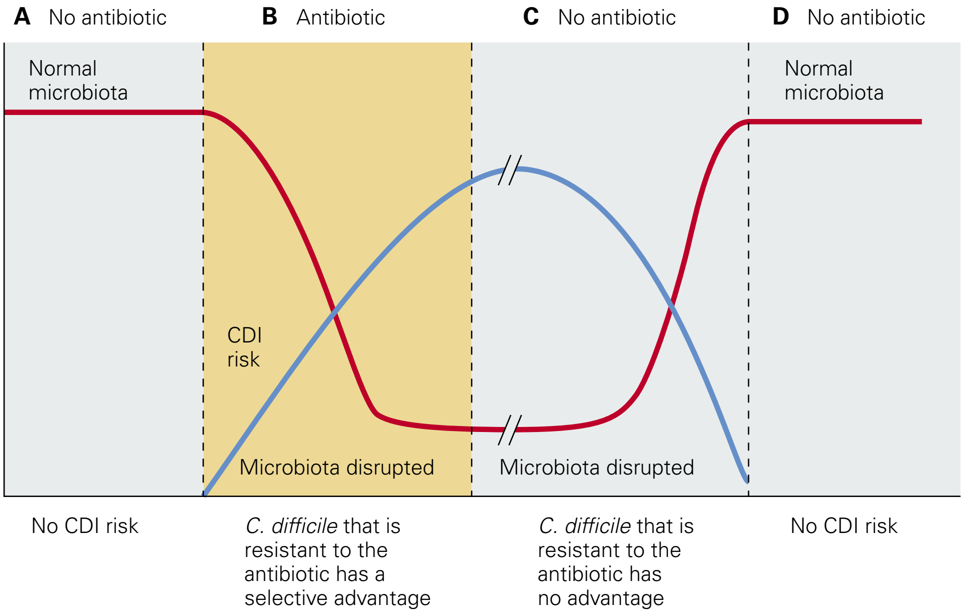


Figure 18-13. The effects of antibiotics on the normal gut microbiota and the risk of *Clostridium difficile* infection. (A) Patients are resistant to *C. difficile* infection (CDI) if their normal gut microbiota is not disrupted by antibiotics. **(B)** Once antibiotic treatment starts, infection with a *C. difficile* strain that is resistant to the antibiotic is more likely while the antibiotic is being administered owing to the presence of the antibiotic in the gut. **(C)** When the antibiotic treatment stops, the levels of the antibiotic in the gut diminish rapidly, but the microbiota remains disturbed for a variable period of time (indicated by the break in the graph), depending on the antibiotic given. **(D)** During this time, patients can be infected with either resistant or susceptible *C. difficile*. Finally, after the microbiota recovers, colonization resistance to *C. difficile* is restored. Adapted from Rupnik M, Wilcox MH, Gerding DN. 2009. *Nat Rev Microbiol* **7**:526–536, with permission.

of antibiotic in the intestine fall, *C. difficile* is able to resume growth faster than most other colonic bacteria such that it repopulates the colon before the normal microbiota is able to reestablish itself. Although many strains of *C. difficile* are scored "susceptible" using the standard cutoff between resistant and susceptible strains, they are less susceptible than many of the major groups of colonic bacteria. A small difference in antibiotic susceptibility as measured by *in vitro* tests could give bacteria a huge edge in the colon.

Normally, less than 5% of people in a healthy population harbor *C. difficile* in their intestinal tracts; however, in hospitals, where the bacteria can spread from one patient to another, this percentage rises to around 20%. In some extreme cases, such as nursing homes, over 50% of the residents may be asymptomatically colonized by *C. difficile*. Risk of colonization is linked directly to the length of time spent in the hospital, particularly if in hospitals that have experienced outbreaks of *C. difficile* disease in the past. It is also important to note that even in people who are colonized with *C. difficile* and who are treated with the antibiotics associated with pseudomembranous colitis, only a subset of individuals actually develops the disease (Figure 18-14). Some people become colonized with nontoxigenic strains of *C. difficile* that do not produce the clostridial toxins (see chapter 12), while others who are colonized by toxigenic strains seem to mount a sufficient humoral immune response against the toxins to remain asymptomatic. Additionally, it appears that certain people are more likely to develop CDIs because of differences in the composition of their colonic microbiota, while in others it is because they are physiologically more susceptible to the effects of the clostridial toxins that mediate the disease.

C. difficile is a strict anaerobe and dies rapidly outside the colon. Thus, at first glance, it might appear unlikely that *C. difficile* would be transmissible from person to person. However, *C. difficile* can form spores, which can not only

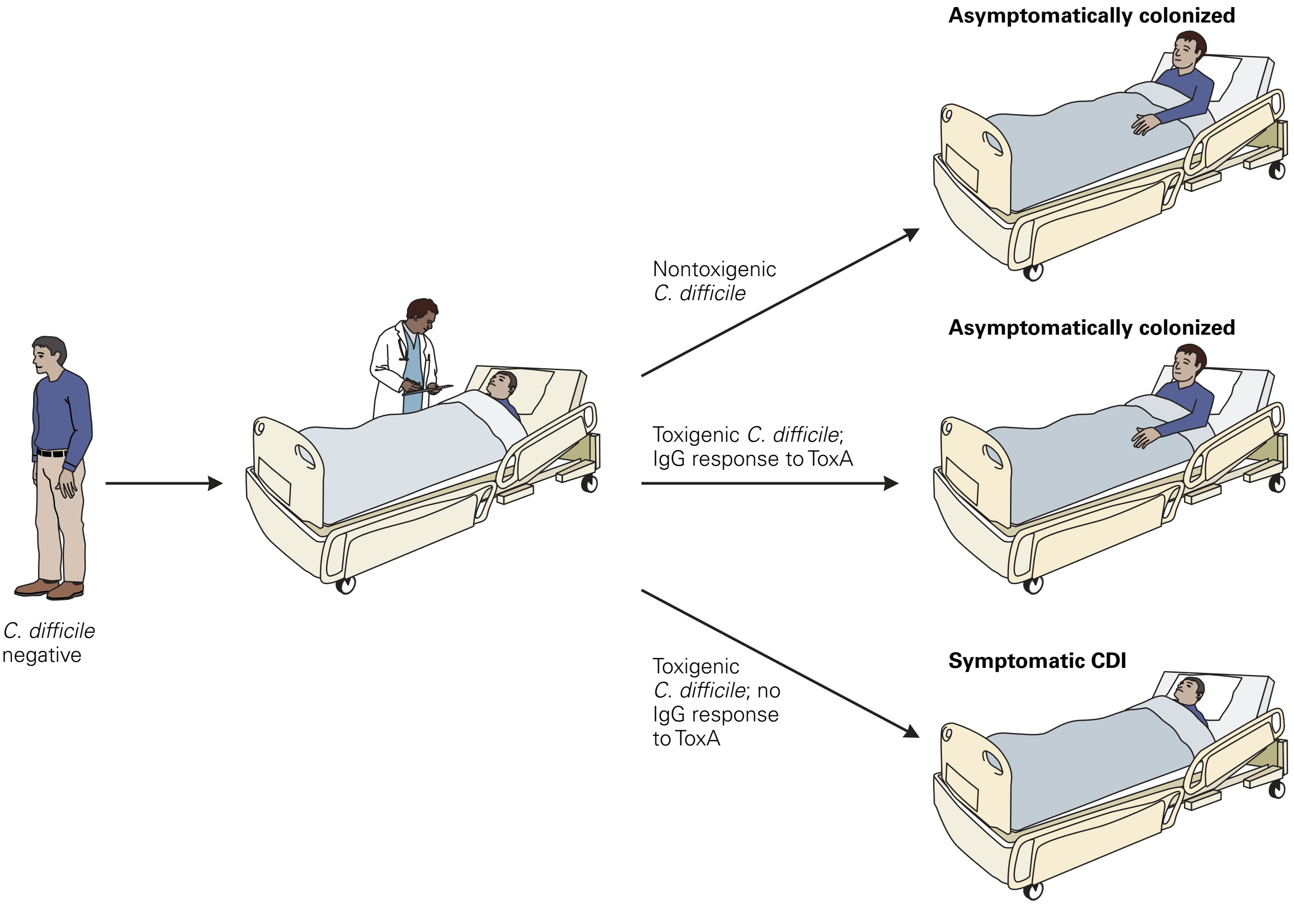

Asymptomatically colonized
Nontoxigenic *C. difficile*
Asymptomatically colonized
Toxigenic *C. difficile*; IgG response to ToxA
C. difficile negative
Symptomatic CDI
Toxigenic *C. difficile*; no IgG response to ToxA

Figure 18-14. Model for the acquisition of *Clostridium difficile* infection. Patients are exposed to *C. difficile* spores through contact with the hospital environment or health care workers. After taking an antibiotic, they develop CDI if they acquire a toxigenic *C. difficile* strain and fail to mount a protective serum IgG antibody response to the toxin TcdA; if they can mount an antibody response, they become asymptomatically colonized with *C. difficile*. If they acquire a nontoxigenic *C. difficile* strain, they also become asymptomatically colonized. Colonized patients have been shown to be protected from CDI. Adapted from Rupnik M, Wilcox MH, Gerding DN. 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Nature Rev Microbiol* **7**:526–536, with permission.

persist in the environment for many months but can survive passage through the stomach when ingested. The spread of spores from a colonized person to the surrounding environment is facilitated by the fact that an early symptom of pseudomembranous colitis is diarrhea, a notorious source of aerosols and fecal contamination. Members of the hospital staff who care for patients colonized with *C. difficile* also transmit the spores to other patients on their hands and clothing. Even apparently innocuous practices, such as reuse of handles on electronic thermometers, have been found to spread *C. difficile* spores. Additionally, colonization of an otherwise healthy person with *C. difficile* causes no apparent symptoms as long as the clostridia are present in low concentrations further adding to the difficulty of containing its spread.

Virulence factors. In early attempts to predict which patients were at highest risk for developing pseudomembranous colitis, attention focused on patients who entered the hospital already colonized with *C. difficile*. However, careful studies of these patients revealed that they were, if anything, less likely to develop pseudomembranous colitis than patients who acquired the bacteria in the hospital, though exactly why is still not clear. Possibly, *C. difficile* strains that survive in the hospital environment are more resistant to antibiotics than strains colonizing people in the community. Alternatively, there may be some partial immunity to *C. difficile* disease in cases where people have carried the bacteria asymptomatically for a long period of time.

A model for the contribution of the TcdA and TcdB toxins to the development of CDI and mucosal damage is shown in Figure 18-15. TcdA activates neurons and attracts and activates neutrophils (PMNs) by stimulating intestinal mucosal cells to produce cytokines and other proinflammatory proteins. Activation of the enteric neurons affects the motility of intestinal contents and may contribute to diarrhea. Neutrophils, moving into the area create an inflammatory response, which contributes to mucosal cell destruction. Additionally, neutrophils migrating between mucosal cells disrupt the tight junctions that normally prevent fluids from flowing across the mucosal membrane, which not only allows water to leak from tissue into the lumen of the intestine but also allows TcdB to cross the mucosal membrane by diffusion. TcdB can then damage the tissue underlying the mucosal membrane, further contributing to damage of the intestinal wall. If damage becomes too extensive, colonic Gram-negative bacteria or even just their LPS can breach the intestinal wall, enter the bloodstream, and cause septic shock. Also, the extensive tissue damage caused by the toxins elicits a strong inflammatory response that can contribute to septic shock.

The first step in virulence is adherence to mucin and the cells that line the colon. Several surface proteins have been proposed as adhesins, including surface layer (S-layer) proteins, such as SlpA, fibronectin-binding adhesins, flagellar components, and other cell wall-binding proteins. However, CDI symptoms are caused by the clostridial toxins produced following colonization. These toxins damage the colonic mucosa resulting in accumulation of fibrin, mucin, and dead host cells, which form a characteristic yellowish layer on the surface of the colon. Damage caused by these toxins also provokes an inflammatory response that recruits neutrophils to the area and to the lesions. Initially, numerous separate lesions appear, covered by patches of debris. Eventually, these scattered lesions coalesce, and a sheet-like layer of debris covers a larger area of the colon surface. This layer of cellular debris (**pseudomembrane**) distinguishes the disease from other types of intestinal inflammation (**colitis** or **enterocolitis**) and gives the disease its name. Untreated pseudomembranous colitis can be fatal, with the symptoms similar to those seen in cases of septic shock.

C. difficile strains that cause pseudomembranous colitis produce two large AB-type protein toxins, **toxin A (TcdA)** and **toxin B (TcdB)**, which are thought to be responsible for the symptoms of CDIs. These toxins act by modifying host cell G proteins that control many cellular activities, including actin polymerization. TcdA and TcdB are large single-polypeptide, modular bacterial toxins with molecular masses of 308 kDa and 270 kDa, respectively, and are similar in amino acid sequence (45% identity). The *tcdA* and *tcdB* genes are located on a pathogenicity island called **PaLoc** (for pathogenicity locus) (Figure 18-16A). Regulatory genes, *tcdC* and *tcdR*, are located in the same region. A small open reading frame between *tcdB* and *tcdA* encodes a protein with sequence similarity to bacteriophage release proteins, called **holins**. PaLoc is present in all toxigenic strains of *C. difficile* and is replaced by a short spacer in nontoxigenic strains.

The structures of the *C. difficile* toxins are illustrated in Figure 18-16A**.** The toxin is comprised of a binding domain that binds to the host-cell receptor, a translocation domain that mediates translocation of the enzymatic portion into the cell cytoplasm, and the catalytic domain that glucosylates (i.e., adds a glucose moiety to) target G proteins. The mechanism for cellular intoxication for TcdA and TcdB is shown in Figure 18-16B. The host cell surface receptor for TcdA appears to be a trisaccharide in animals, but in humans a host surface glycoprotein called GP96, which is found on the apical side of colonic cells. In contrast, TcdB binds to two receptors, chondroitin sulfate proteoglycan 4 (CSPG4) and Frizzled (FZD), found on the basolateral side of the epithelial cell layer. Given the different localizations of the receptors of the two toxins, it is likely that one of the roles of TcdA toxicity is to disrupt tight junctions to allow egress of TcdB to the basolateral membranes of the host cells. After receptor binding, the toxins are endocytosed into the cells, and the catalytic glucosyltransferase domain (GTD) is then translocated from the endosome into the cytosol through a pH-dependent process, where the GTD is proteolytically cleaved by its cysteine protease, autoprocessing domain (APD), and released.

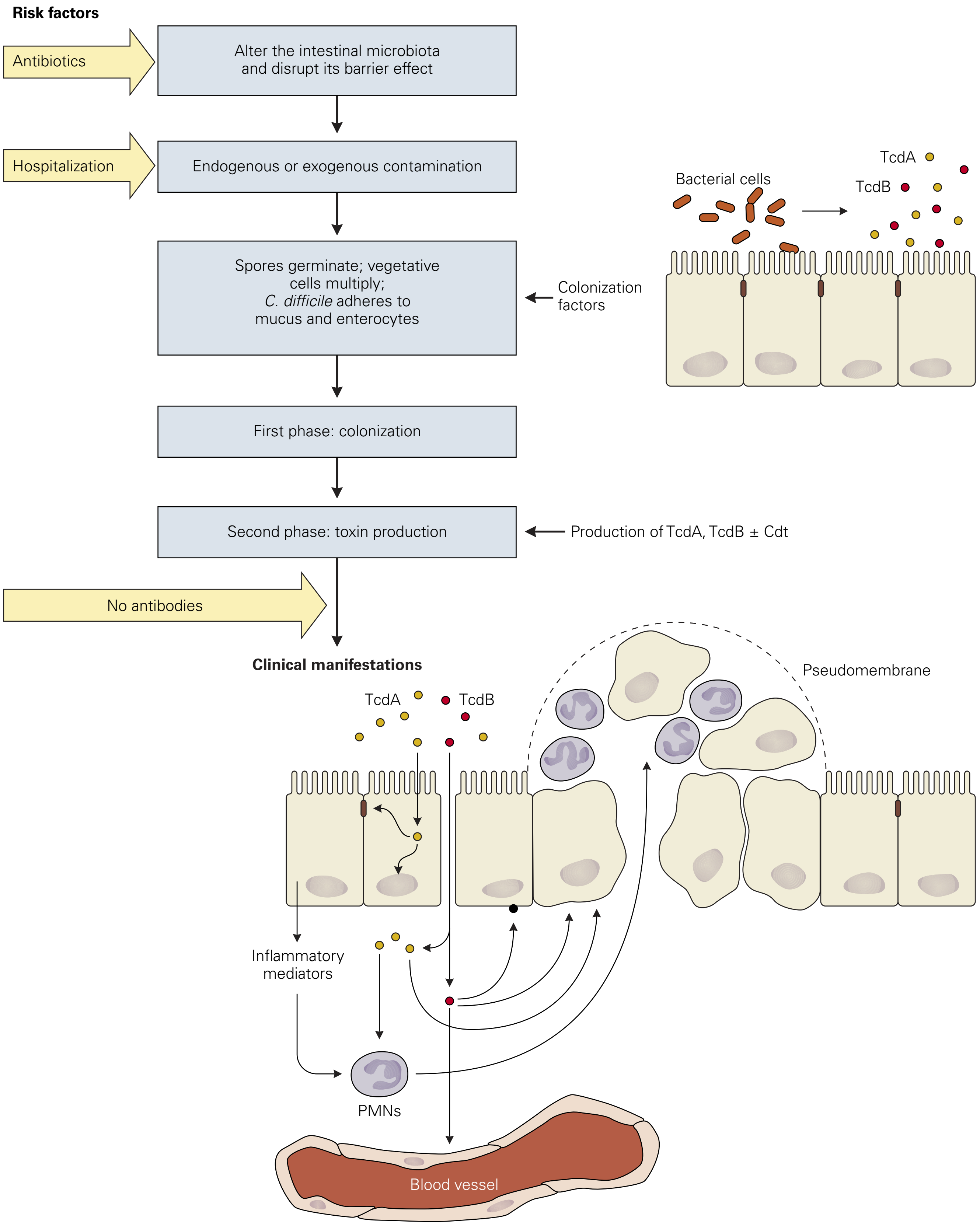

Risk factors
Antibiotics
Alter the intestinal microbiota and disrupt its barrier effect
Hospitalization
Endogenous or exogenous contamination
Spores germinate; vegetative cells multiply; *C. difficile* adheres to mucus and enterocytes
Colonization factors
Bacterial cells
TcdA
TcdB
First phase: colonization
Second phase: toxin production
Production of TcdA, TcdB ± Cdt
No antibodies
Clinical manifestations
TcdA
TcdB
Pseudomembrane
Inflammatory mediators
PMNs
Blood vessel

Figure 18-15. **Steps in the development of CDI and pseudomembranous colitis.** *C. difficile* colonizes the intestine (colon) after disruption of the normal intestinal microbiota by antibiotic treatment. After endogenous or exogenous contamination by *C. difficile* spores, spores germinate and vegetative forms multiply and then adhere to the mucus layer to colonize. The bacteria then produce the main virulence factors, the two large clostridial toxins A and B (TcdA and TcdB), which enter host cells and inactivate small Rho GTPases involved in cytoskeletal function through glucosylation. Some strains also produce another AB toxin (CdtAB) that ADP-ribosylates actin. TcdA binds to the apical side of the cell and, after internalization, causes cytoskeletal changes that result in disruption of tight junctions and loosening of the epithelial barrier, in cell death, or in the production of inflammatory mediators that attract neutrophils. Disruption of tight junctions enables both TcdA and TcdB to cross the epithelium. TcdB binds preferentially to the basolateral cell membrane. TcdB also enters the bloodstream and appears to have a tropism for cardiac tissue. Both toxins induce the release of various immunomodulatory cytokines from epithelial cells, phagocytes, and mast cells, resulting in inflammation, recruitment of neutrophils and formation of the pseudomembrane. Adapted from Deneve C, Janoir C, Poilane I, Fantinato C, Collignon A. 2009. *Int J Antimicrob Agents* **33 Suppl 1:**S24-28, and from Rupnik M, Wilcox MH, Gerding DN. 2009. *Nat Rev Microbiol* **7:**526–536, with permission.

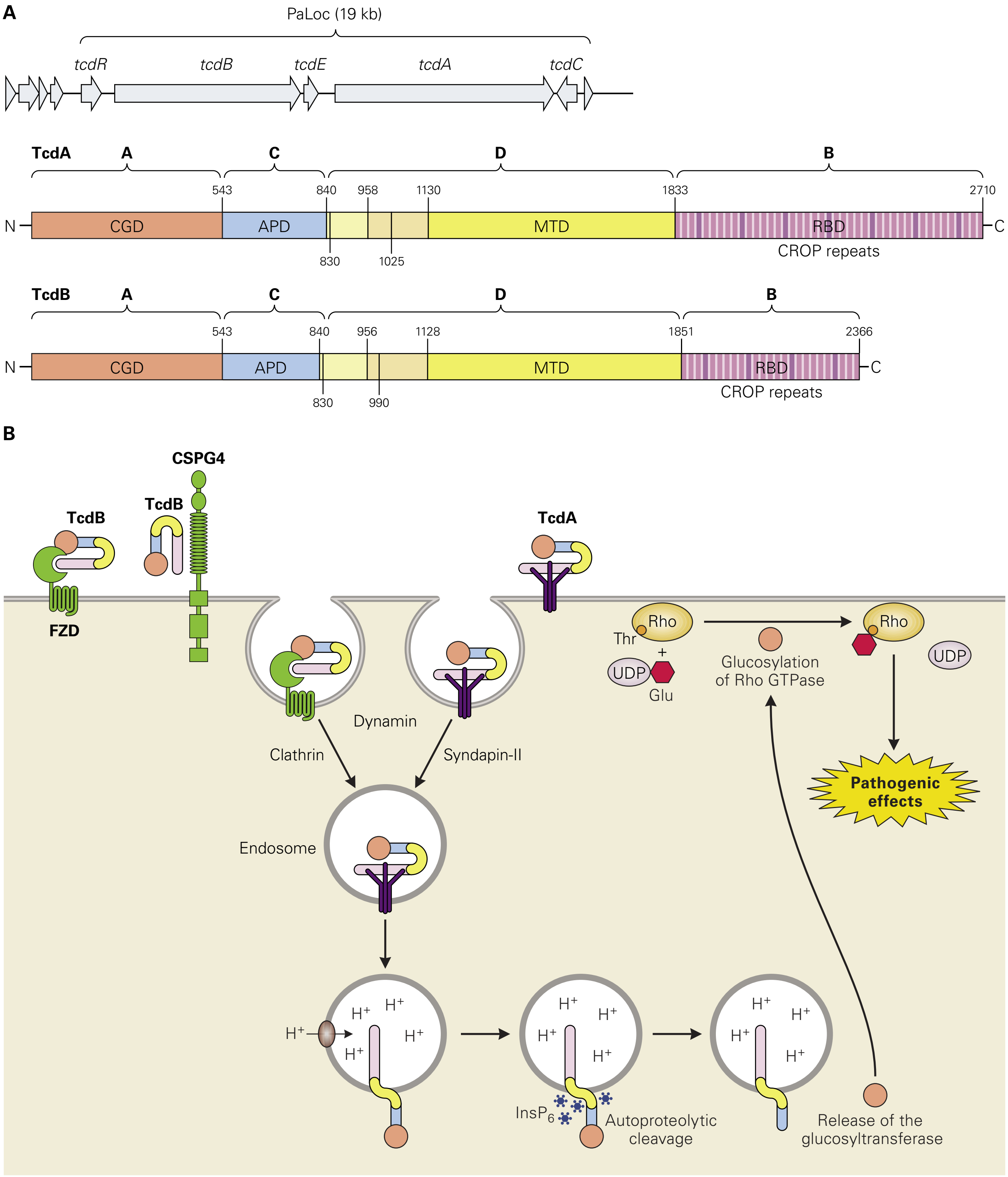

A
PaLoc (19 kb)
tcdR
tcdB
tcdE
tcdA
tcdC
TcdA
A
C
D
B
543
840
958
1130
1833
2710
N
CGD
APD
MTD
RBD
C
830
1025
CROP repeats
TcdB
A
C
D
B
543
840
956
1128
1851
2366
N
CGD
APD
MTD
RBD
C
830
990
CROP repeats
B
CSPG4
TcdB
TcdB
TcdA
FZD
Rho
Thr
+
UDP
Glu
Glucosylation
of Rho GTPase
Rho
UDP
Pathogenic
effects
Dynamin
Clathrin
Syndapin-II
Endosome
H+
H+
H+
H+
H+
H+
H+
H+
H+
H+
H+
H+
H+
H+
InsP6
Autoproteolytic
cleavage
Release of the
glucosyltransferase

Figure 18-16. **Toxins produced by *Clostridium difficile* and their effects on G proteins. (A)** Toxigenic strains of *C. difficile* that cause CDI produce two main virulence factors, the two large clostridial toxins A and B (TcdA and TcdB), that enter host cells and inactivate small Rho GTPases involved in cytoskeletal function through glucosylation. The genes for the toxins are encoded on the pathogenicity locus (PaLoc), which is comprised of five genes. Both toxins are single-chain proteins, and several functional domains and motifs have been identified. The protein domain organizations of TcdA (308 kDa) and TcdB (270 kDa) are shown in detail below the PaLoc. Their structures consist

The GTDs of both toxins have the same mechanism of action: they are UDP-glucosyltransferases that transfer a glucose moiety from UDP-glucose to a threonine residue in their target G-protein substrate, **small Rho GTPases**, including **Rho**, **Rac**, and **Cdc42**. TcdA has additional G protein targets, including the small Rap GTPases. All of these targets are important regulatory proteins that control the polymerization and depolymerization of actin in mammalian cells. Small G proteins cycle between an inactive GDP-bound state and an active GTP-bound state. The GTP-bound form mediates the downstream signaling effects of the G protein. The toxins preferentially glucosylate the GDP-bound form, which reduces the GTPase activity of the protein. The modification also interferes with the interaction between the G protein and its downstream effectors, thereby blocking Rho-dependent cell signaling and causing disruption of actin cytoskeleton, which leads to cell rounding and other cytopathic and cytotoxic effects.

Toxin levels are highest in the medium of late-exponential and stationary-phase cultures. TcdR is an alternative sigma factor that mediates transcription of the *tcdBEA* operon. TcdC is an antisigma factor for TcdR that antagonizes TcdR function during exponential growth. Growth phase is not the only signal sensed by the toxin regulatory genes. Stresses of various types, including limitation for carbon and nitrogen and oxygen, also increase toxin production. These stresses may be linked because one effect of oxygen on an obligate anaerobe is to reduce metabolic activities, causing a greater reliance on exogenous amino acids and other nutrients. Recently, the global repressor CodY, which senses the cell's metabolic state by responding to intracellular concentrations of branched-chain amino acids (i.e., isoleucine, leucine, and valine) and GTP, was shown to regulate toxin gene expression.

Strains of *C. difficile* vary considerably in their level of TcdA and TcdB production, but toxigenic strains almost always produce both TcdA and TcdB and at roughly equivalent levels. This observation is not too surprising, given that the two toxin genes are co-transcribed. In some strains, the levels of these toxins can be quite high. Under *in vitro* conditions that give maximum toxin production, the toxins can account for up to 5% of total protein! Epidemic hypervirulent strains that have recently emerged produce at least 15-fold more TcdA and TcdB than previous strains. Toxin production in these strains seems to be completely deregulated and is produced during both the exponential and stationary phase. This massive increase in toxin production is correlated with a frame-shift mutation that inactivates the TcdC antisigma factor that normally negatively regulates toxin production during the exponential phase.

Some *C. difficile* strains also produce a third AB-type toxin, called **binary toxin (CDT)**, which also modifies actin (Figure 18-17). Considerably less is known about CDT binary toxin, which is present in fewer than 10% of *C. difficile* strains. This AB-type toxin is comprised of a catalytic subunit (CdtA) and a receptor-binding/translocation subunit (CdtB). The CDT toxin is encoded by the CDT locus located in a different region of the chromosome from PaLoc. This locus also encodes a positive regulator, CdtR, of the *cdtAB* genes, which likely acts as a response regulator of a two-component system, although no cognate histidine sensor kinase gene is encoded in the locus. CdtB targets the CDT to colon epithelial cells, where CdtA is translocated into the cytosol and ADP-ribosylates actin molecules leading to cytoskeleton disorganization. One function of the CDT toxin may be to potentiate the toxicity of TcdA and TcdB, thereby causing more severe diseases in hypervirulent strains.

Prevention and treatment of CDI. So far, the most effective efforts to prevent pseudomembranous colitis have been to restrict the use of the antibiotics most likely to cause it, coupled with increased hygiene practices to minimize contamination. However, one approach will not work without the other. Careful cleaning of rooms where patients with pseudomembranous colitis have stayed can reduce, but not eliminate the number of spores in the environment

Figure 18-16. *continued*

of four functional domains: The A domain is the catalytic glucosyltransferase domain (GTD) that transfers glucose groups from UDP-glucose to Thr residues of Rho GTPases. The C domain is the autoproteolytic processing domain (APD) that consists of a cysteine protease that is activated by cytosolic inositol hexakisphosphate ($InsP_6$) to cleave the toxin and release the GTD into the cytosol. The D domain is involved in membrane translocation and delivery (MTD) of the A and C domains into the cytosol. The B domain is the receptor-binding domain (RBD) comprised of repetitive oligopeptides called CROPS. **(B)** Mechanism of cellular intoxication and Rho glucosylation by TcdA or TcdB. TcdA binds to surface sugar groups and gp96 as coreceptors (purple), which triggers cellular uptake into early endosomes via dynamin and syndapin-II. TcdB can bind to two receptors, either through its CROPS domain to the G-protein–coupled receptor Frizzled (FZD) or through its non-CROPs part of the B domain to chondroitin sulfate proteoglycan 4 (CSPG4). In each case, binding triggers cellular uptake into early endosomes via dynamin and clathrin. In both cases, the toxins reach an acidic late endosome, where the GTD and APD domains are then translocated across the endosomal membrane into the cytosol. Cytosolic inositol hexakisphosphate (InsP6) activates the protease activity of APD, which then cleaves and releases the GTD into the cytosol. GTD then moves to the cell membrane, where it gains access to its Rho G-protein substrate. Normal cycling of membrane-bound Rho G proteins between the GDP-bound form and the GTP-bound form (top) is disrupted by glucosylation of a Thr residue, which is buried in the GTP-bound form, but becomes exposed on the surface of the GDP-bound form. Glucosylation of Rho proteins by GTD prevents interaction with downstream effectors, which results in cytopathic or cytotoxic effects. Binding of TcdB to FZD also activates cellular signaling through Dishevelled (Dvl), which in turn activates the Rho, Rac, and β-catenin signaling pathways. Adapted from Aktories K, Schwan C, Jank T. 2017. *Annu Rev Microbiol* **71:**281-307, with permission.

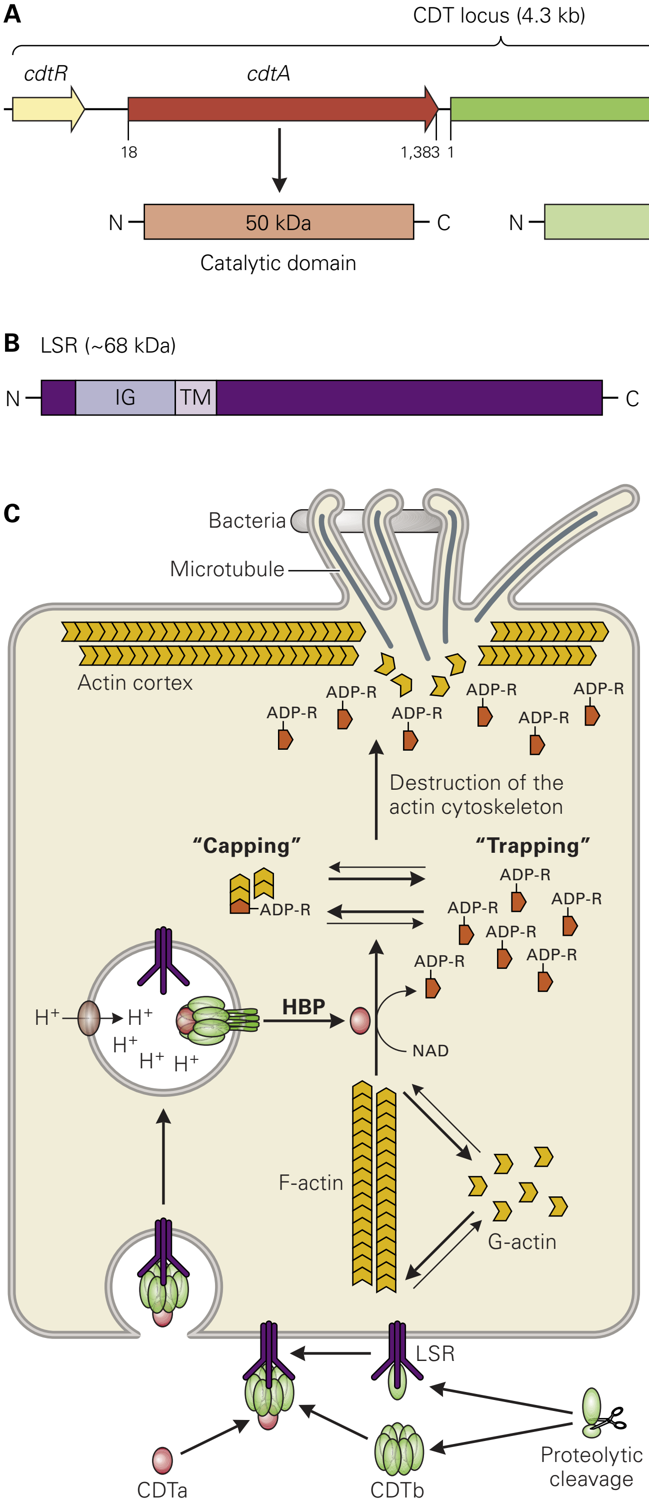

A
CDT locus (4.3 kb)
cdtR
cdtA
cdtB
18
1,383
1
2,631
N
50 kDa
C
Catalytic domain
N
100 kDa
C
Translocation and binding domain
B
LSR (~68 kDa)
N
IG
TM
C
C
Bacteria
Microtubule
Actin cortex
ADP-R
Destruction of the actin cytoskeleton
"Capping"
"Trapping"
ADP-R
H+
H+
H+
H+
H+
HBP
NAD
F-actin
G-actin
LSR
CDTa
CDTb
Proteolytic cleavage

Figure 18-17. Other toxins produced by *Clostridium difficile*. Some hypervirulent strains of *C. difficile* also produce another AB toxin (CdtAB) that ADP-ribosylates actin and potentiates the effects of TcdA and TcdB. **(A)** The genes are encoded on a separate region of the chromosome (CdtLoc) and are comprised of three genes. The binary toxin is composed of two unlinked proteins, CdtB and CdtA. CdtB has a binding function and CdtA is the enzymatic component. Adapted from Rupnik M, Wilcox MH, Gerding DN. 2009. *Nat Rev Microbiol* **7:**526–536, with permission. **(B)** Lipolysis stimulated lipoprotein receptor (LSR) is the target receptor for CDT. The extracellular domain has an immunoglobulin-like structure. **(C)** Mechanism of action of CdtAB. The proteolytically activated binding subunit (CDTb) binds to the cell surface receptor LSR and forms heptamers. The catalytic subunit (CDTa) forms a complex with the heptamer and is endocytosed. Endosomal acidification triggers insertion of the heptamers into the membrane to form pores and CDTa is translocated into the cytosol with the assistance of host cell heat shock protein (HSP). CDTa ADP-ribosylates actin, preventing it from polymerizing. Depolymerization of cortical actin, which is located beneath the cell membrane, causes formation of long microtubule-based protrusions, which in turn enhance the adherence and colonization of the clostridia. Adapted from Gerding DN, Johnson S, Rupnik M, Aktories K. 2014. *Gut Microbes* **5:**15-27, with permission.

Successful treatment of CDI depends on early diagnosis. Patients receiving antibiotics known to cause pseudomembranous colitis are watched carefully for signs of diarrhea. In many U.S. hospitals, nearly every patient with antibiotic-associated diarrhea is tested for the presence of TcdA and TcdB in their feces. If pseudomembranous colitis is suspected, the patient is taken off of the antibiotic that precipitated the disease and given vancomycin or metronidazole, two antibiotics that are still effective against *C. difficile*. Timely administration of oral vancomycin or metronidazole is usually successful in preventing the development of full-blown pseudomembranous colitis. If this treatment is successful and the patient recovers, there is still the chance of a relapse once the therapy is discontinued. Relapses occur in as many as 20% of cases, and multiple relapses in the same individual are common. Presumably, relapses are due to failure to fully clear *C. difficile* and to restore a stable, nonpathogenic microbiota.

One initial strategy to treat recurrent pseudomembranous colitis was to restore the resident microbiota by giving the patient an enema with dilute feces taken from family members. Although an enema does not reach very high into the colon and thus might not seem to be an effective strategy for repopulating the entire colon, this treatment has been effective at preventing relapses. Another approach has been to replenish the colonic microbiota with probiotic nonpathogenic bacterial and yeast species, such as *Saccharomyces boulardii* or *Bifidobacteria*. To date, convincing data are lacking for the effectiveness of probiotic administration to prevent or treat CDIs. By comparison, the most effective treatment for *C. difficile*-mediated pseudomembranous colitis that is in current use involves **fecal microbiota transplantation** (**FMT**). Numerous studies over the past 15 years have reported success rates of up to 90% for treating CDI, particularly for cases of recurring CDI. There are a variety of methods used to deliver the fecal matter, including oral routes via capsules, duodenal infusion via nasogastric tube or endoscopy, and rectal routes via enema or colonoscopy (Figure 18-18). So far, the biggest concerns about use of FMT to treat CDI are safety—the inherent risk for adverse outcomes associated with transfer of live bacteria, along with other microbial and host components, present in a fecal sample from a healthy donor to the gastrointestinal tract of a vulnerable recipient. Protocols and policies for screening donors and processing and testing potential FMT procedures and practices are still undergoing extensive study and debate.

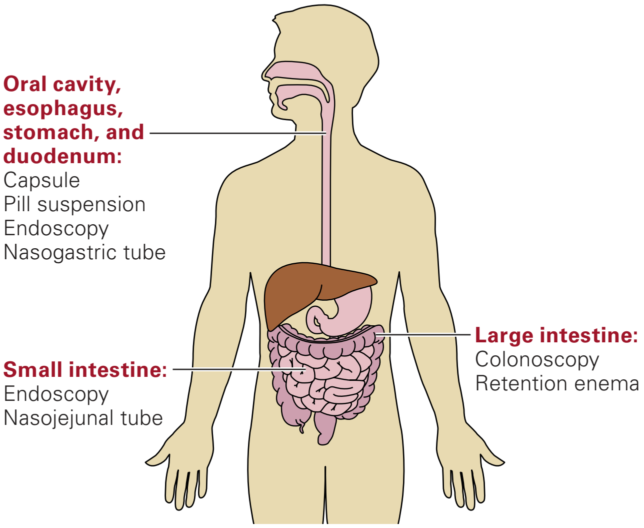


Figure 18-18. Routes of administration for fecal microbiota transplantation. The route chosen for delivery of FMT will depend on the source of the microbiota that is transplanted, the overall health of the patient, and the disease being treated (CDI versus recurrent CDI or other enterocolitis).

Other Gram-Positive Opportunists

Although we only presented a small sampling of different pathogens, there are a number of other important Gram-positive opportunists. *Streptococcus pyogenes* (Group A Strep), which is a commensal found in the throat and saliva of about 30% of people, causes a range of serious diseases ranging from pharyngitis (the well-known "strep throat") to endocarditis and necrotizing fasciitis (the feared "flesh-eating" disease). Similar to *S. aureus*, *S. pyogenes* is a diverse group of bacteria that have an astounding capacity to regulate and produce various adhesins and toxins. In fact, we used this regulation as the paradigm for thinking about the sequential expression of genes as bacterial pathogenesis progresses from colonization to persistence to spread (Figure 11-1). Rather astoundingly, *S. pyogenes* is one of the few commensal species that has not acquired multidrug resistance and remains sensitive to β-lactam antibiotics.

Analogous to *S. pneumoniae*, *Streptococcus agalactiae* (Group B Strep) is a common commensal bacterium with a high potential to cause a variety of invasive diseases. *S. agalactiae* is a member of the vaginal microbiota in about 30% of women, where it can potentially spread to newborn infants and cause life-threatening diseases. *S. agalactiae* is also emerging as a serious antibiotic-resistant pathogen of adults, especially those at risk for invasive pneumococcal diseases. Like *S. pneumoniae*, *S. agalactiae* is equipped with a set of colonization and virulence factors, including a sialic acid-rich capsular polysaccharide, pore-forming toxins, a hyaluronate lyase, adhesins that bind to the extracellular matrix, D-alanine decoration of LTA, and peptidases and proteases that interfere with the innate immune response.

Finally, similar to *S. epidermidis*, *Enterococcus faecium* has emerged as a serious cause of hospital-associated bacteremia, urinary tract infections, wound infections, and catheter-associated infections. This ubiquitous commensal bacterium from the gastrointestinal tracts of humans and livestock, including poultry, was once thought to be benign. However, in the past 20 years, *E. faecium* has rapidly acquired antibiotic resistance mechanisms, including resistance to vancomycin (see chapter 16), which make it extremely difficult to treat once infection has occurred. In fact, hospital isolates of *E. faecium* seem to be evolving rapidly and have acquired accessory genes that are not present in gut isolates. *E. faecium* seems to be exhibiting the disturbing trend noted numerous times in this chapter of diversifying its genome to become more resistant to antibiotics and more virulent to human hosts.

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Questions

1. Describe how understanding the mechanisms involved in bacterial adhesion to host cells might lead to the development of new preventive therapies or measures.
2. Critique the statement: Members of the same bacterial species are usually found at the same anatomical sites of the host body.
3. What components of the Gram-positive cell wall are responsible for septic shock?
4. Superantigens are able to elicit toxic effects throughout the body. Describe how superantigens are able to initiate these effects and how this might be an advantage to the infecting bacteria.
5. On one particularly busy evening, you forget to put a casserole in the refrigerator after dinner. Even though the food has not been refrigerated, it still “looks” okay and you decide to eat the casserole the next day.
    a. If the casserole is contaminated with *Staphylococcus aureus*, will reheating protect you from food poisoning? Provide your rationale.
    b. If the casserole is contaminated with *Clostridium botulinum*, will reheating protect you from food poisoning? Provide your rationale.
6. Staphylococcal enterotoxin and toxic shock syndrome toxin act as ____________________, crosslinking cells of the innate immune system, and are potent nonspecific activators of _________________________.
7. *Streptococcus pneumoniae* depends on ____________ and _________________ to evoke the inflammatory response for tissue damage and entry into the bloodstream.
8. *Staphylococcus aureus* uses a ____________________ system to regulate its virulence factors: _____________ are expressed during early growth phases and then downregulated as _________________ are upregulated when high density is reached.
9. You are characterizing a mutant of *Bacillus subtilis*. The mutation affects the cell wall such that the cells make only a thin layer of peptidoglycan (making up about 20% of the cell wall). What would you expect the Gram stain reaction to be?

Solving Problems in Bacterial Pathogenesis

1. MRSA is resistant to several commonly prescribed antibiotics that are usually effective against Gram-positive bacteria (methicillin, penicillin, and cephalosporins), and an infection with a MRSA strain can be deadly if left untreated. MRSA is subcategorized as community-associated (CA)-MRSA or hospital-associated (HA)-MRSA, depending on how the infection is usually acquired. Most CA-MRSA are still sensitive to many antibiotics such as trimethoprim, tetracycline, and clindamycin, but HA-MRSA are often resistant to these drugs, while still sensitive to vancomycin and linezolid. Although most CA-MRSA bacteria are not as resistant to antibiotics as the HA-MRSA strains, the recent strains that we are hearing about in the news appear to be more virulent. Most MRSA strains produce type CP5 or CP8 polysaccharide capsules, surface-exposed protein A, and a number of toxins, superantigens, and proteases. MRSA strains producing CP5 show a significantly higher bacteremia level than strains producing CP8. A group of researchers have recently discovered that bacterial cultures of a particularly virulent strain of CA-MRSA (called USA300) cause apoptosis in leukocytes and neutrophils, as evidenced by release of cytochrome c from mitochondria, followed by cytokine release and cell death.
    a. Antibodies generated against bacterial culture medium from the USA300 strain identified two proteins of 33 and 44 kDa on Western blots, named LukS and LukF, respectively. To determine the localization of these two proteins researchers performed SDS-PAGE and Western blot analysis of lysates from the bacterial cell pellets and the bacterial culture medium as well as lysates of human neutrophils treated with the USA300 strain. The results from Western blot analysis are shown in the left panel of Figure 1. The researchers then applied the cell-free bacterial culture medium to the neutrophils and performed subcellular fractionation of the treated neutrophils. The results from Western blot analysis are shown in the right panel of Figure 1. Provide an interpretation (with rationale) of the results shown in Figure 1.
    b. The researchers speculate that LukS and LukF might be two subunits of an AB-type toxin. Based on the results shown in Figure 1, do you agree with this interpretation? Provide your rationale. Provide an experiment that would allow you to verify your answer.
    c. The researchers suspect that LukF might bind to specific protein receptors on neutrophils. Describe an

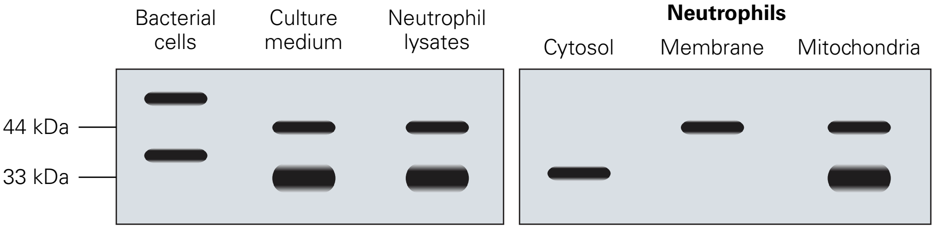

Figure 1. **Western blots using antibodies against USA300 secreted proteins.**

experiment they might perform to isolate and identify this receptor using the antibodies that recognize LukF.

d. The researchers suspect that LukS and LukF are responsible for the necrotizing lung and tissue damage observed during severe infection with CA-MRSA. What four criteria must the researchers satisfy to establish this connection? Provide at least two additional modern molecular experiments (different from those already described) that could be performed to help satisfy these criteria for this connection.

e. Using Western blot analysis, the researchers discover that the two proteins are not made until the late-logarithmic or stationary phase. When they take medium from overnight bacterial cultures and add it to fresh bacterial cultures, they find that they can stimulate production of the proteins much sooner. Provide a possible explanation for this observation.

f. When the researchers purify the proteins from culture media and perform N-terminal sequencing of the proteins, they discover that the proteins are encoded by a two-gene operon, which they name *lukSF*. Describe a strategy that the researchers could use to identify potential regulatory factors that affect the expression of the two proteins encoded by *lukSF*. Be sure to provide the reagents, screening conditions, how they would visualize/measure the results, and how they would verify the results.

g. The researchers propose that development of a two-component vaccine made up of LukS or LukF in combination with CP5 or CP8 would be effective protection against CA-MRSA. What led the researchers to propose this? Provide at least two possible reasons.

h. Which two of the four possible vaccine targets (LukS, LukF, CP5, CP8) would be most effective at generating protective immunity as part of a two-component vaccine against CA-MRSA? Be sure to provide your rationale. Describe how these two components plus at least one additional component could be used to make an "ideal" multicomponent vaccine. Be sure to provide a rationale for your choice of third component and overall vaccine design.

i. Provide a mechanism for how protein A contributes to virulence (i.e., what is its function?).

j. Provide a mechanism for how the superantigens contribute to MRSA virulence. What is a potential long-term consequence of infection with MRSA strains that produce superantigens?

k. Describe how you might identify those genes that are unique to CA-MRSA (versus HA-MRSA) using comparative genome sequencing. How could you determine which of those unique genes encode antigens that generate protective antibodies in patients?

2. In your capacity as an expert working for the Centers for Disease Control and Prevention, you were asked to investigate a disease outbreak at several hospitals in the Washington, DC area, where the patients died after undergoing surgery. You subsequently identified the pathogen as being closely related to *Clostridium perfringens*, which is a Gram-positive anaerobic bacterium that can cause serious human and animal diseases, including clostridial myonecrosis (i.e., "flesh-eating" disease) and gas gangrene (i.e., "tissue-rotting" disease). *C. perfringens* strains are not intracellular pathogens, but are known to produce many extracellular enzymes and toxins, one of which is perfringolysin O (PFO). Being interested in understanding the regulation of these secreted toxins, you performed transposon mutagenesis using a mouse model of gas gangrene infection and isolated a number of avirulent mutants, five of which were in genes unrelated to the toxin genes, yet these mutants no longer produced the toxins, including PFO. Four of the disrupted genes (*mut1*-*mut4*) were found in a single operon, while the other gene (*mut5*) was found upstream of the other genes, but transcribed in the opposite direction. You then made in-frame deletions of each of these genes and designated the resulting mutants as CpMut1-CpMut5. You also cloned each of the genes and found that you could only express four of the corresponding proteins (Mut2-Mut5) in another Gram-positive bacterium, *Bacillus subtilis*, but although the *mut1* gene was transcribed as assessed by Northern blot, you were unable to detect any expressed protein corresponding to

the *mut1* gene. You were interested in characterizing these mutants and determining the functional identity of the proteins, so you designed a series of experiments.

a. In the first experiment, you determined the cellular localization of the proteins encoded by the genes *mut2* through *mut5*. Describe how you conducted this experiment. Be sure to include experimental design, generation of the reagents used, and how you visualized and interpreted the data.
b. In the second experiment, you found that the cellular fractionation results indicated that both CpMut2 and CpMut3 are cytoplasmic proteins, while CpMut4 and CpMut5 are integral membrane proteins. In addition, you found that although all of the mutants are defective in making the toxins, as evidenced by using a reporter gene downstream of the *pfo* gene, when you add cell-free filtrates from overnight cultures of wild-type *C. perfringens* or the mutants CpMut2 or CpMut5 to cultures of CpMut3 or CpMut4, all of the toxins are produced. However, when overnight culture filtrates of CpMut3 or CpMut4 are added to cultures of CpMut2 or CpMut5, no toxin is produced. Provide an explanation for these results. What is the most likely functional identity of each of the proteins encoded by the *mut2-mut5* genes? Provide at least one additional experiment for each of the four proteins that you could perform to confirm your conclusions. (Hint: You should describe at least four separate, different experiments, one for each of the proteins. Be sure to provide the expected results.)
c. For the third experiment, you are now interested in determining the role of the *mut1* gene in virulence. Based on the above information, what is the most likely function of the gene? Be sure to provide your rationale. Design an experiment to confirm your hypothesis.

3. You work for a small biotechnology company interested in developing new vaccines against emerging pathogens. You read about the new pathogen described above in problem 2 and thought it would be a great idea to develop a vaccine against it.

a. What type(s) of immune response would the vaccine need to elicit in order to provide long-lasting protective immunity against each of the pathogens? Be sure to provide your rationale.
b. You propose that CpMut5 and PFO would make excellent targets for development of a two-component recombinant vaccine. Name two scientific arguments that you could provide for your colleagues in support of your proposal.
c. To test the effectiveness of these proteins as antigens, you use a vaccine formulation comprised of formalin-treated, heat-inactivated recombinant CpMut5 and PFO mixed with alum as an adjuvant. You find that after immunization with two boosters you get a strong humoral immune response with high titers of antibodies against both CpMut5 and PFO. However, in challenge studies using the mouse infection models, you find that there is very little protection against the wild-type *Clostridium* strain. Provide an explanation for this result.
d. Considering the above result, provide an alternative strategy (with rationale) that is based on CpMut5 and PFO as antigens to design an optimal, safe recombinant vaccine that does not rely on alum as an adjuvant, but will still lead to robust, long-lasting protective immunity against the pathogen.

19

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CHAPTER 19

The Gram-Negative Opportunistic Pathogens

The opportunists included in this chapter were placed here on the basis of an arbitrary characteristic: their cell wall structure. In fact, Gram-negative bacteria seem to be just as adroit at taking advantage of various breaches in the defenses of the host as Gram-positive bacteria. Perhaps microbiologists should replace the old saying "you can't judge a book by its cover" with "you can't judge an opportunist by its cell wall." Instead, special traits that are at the disposal of individual strains seem to be the critical deciding factors in their ability to rise to the occasion, opportunistically speaking.

Jumping Over the (Cell) Wall: Gram-Negative Bacteria Can Be Opportunistic Pathogens Too!

The list of Gram-negative opportunists now has a large number of entries. Rather than try to cover this increasingly extensive list, examples have been chosen that illustrate some of the different ways in which such Gram-negative bacteria can cause infection. Many of these bacterial species come from the human microbiota, a fact that is not surprising given that the location and constant presence of these bacteria make them best able to take advantage of breaches in the host. Four examples of this type of opportunist, *E. coli, Bacteroides fragilis, Klebsiella pneumoniae,* and *Porphyromonas gingivalis*, exemplify different ways in which such bacteria cause a variety of diseases. Opportunistic Gram-negative pathogens can also come from soil and aquatic environments. Three examples described in this chapter are *Pseudomonas aeruginosa, Burkholderia cenocepacia,* and *Acinetobacter baumannii.* Gram-negative opportunists can even be transmitted by arthropods. Although calling such bacteria "opportunists" may be a bit controversial, they are included here as zoonotic opportunists because they are predominantly animal pathogens that are transmitted occasionally to humans by insect vectors. *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, the only *Ehrlichia* species known to cause disease in humans, serve as examples of this type of pathogen.

Common Traits of Gram-Negative Opportunists

A common trait of opportunists is that they are able to take advantage of certain breaches of human defenses to cause infections that they would normally be unable to cause in the body of a healthy human being. An overview of the opportunists covered in this chapter and the types of opportunities they exploit is provided in Table 19-1. A striking feature of these opportunists is that they do not simply take advantage of any random breach in host defenses. Rather, they seem to be adapted to respond to a

Table 19-1. Examples of breaches in human defenses that are used by opportunists to cause infections

ORGANISM	DISEASE	BREACH IN DEFENSES
E. coli		
K1 strains	Neonatal meningitis	Infants exposed to vaginal bacteria during birth
UPEC	Urinary tract infections	Urinary tract epithelial cell-specific adhesins that help ascend and colonize urinary tract despite urine flow
EIEC	Diarrhea	Evasion of humoral immune responses
K. pneumoniae	Urinary tract infections, septicemia	Evasion of MBL-mediated lectin complement pathway
	Liver abscess	Capsule is antiphagocytic
B. fragilis		
NTBF	Internal abscesses, bacteremia (sepsis)	Lesions in the colon caused by abdominal surgery or other wound trauma
ETBF	Diarrhea, risk factor for colorectal cancer	BFT-mediated loss of intestinal barrier function by disruption of E-cadherin adherens junctions of colonic epithelial cells
P. gingivalis	Periodontal disease, internal infections	Altered complement responses, microbiota dysbiosis and inflammation leading to bone loss, evasion of innate immune defenses
P. aeruginosa	Lung infections in cystic fibrosis patients	Cystic fibrosis causing aberrant mucus flow in lungs
	Wound and burn infections	Bypass innate skin and corneal (eye) defenses
	Ventilator-associated infections	Bypass upper-respiratory defenses
	Catheter-associated infections	Bypass urinary flow defenses
B. cenocepacia	Lung infections	Cystic fibrosis, ventilator pneumonia
A. baumannii	Wound and lung infections	Bypass innate skin and mucosal defenses, respectively
E. chaffeensis	Bacteremia	Arthropod bite directly injects the bacteria into the bloodstream, bypassing innate skin defenses

limited range of opportunities. To a large extent, this adaptation is due to their location relative to a breach in defenses, but it is likely that other characteristics of the specific breaches they favor are responsible for their preferences.

Another trait common to many of them is resistance to a variety of antibiotics (see chapter 16). Of the bacteria covered in this chapter, resistance to multiple antibiotics is particularly striking in *E. coli, B. fragilis, P. aeruginosa, B. cenocepacia, K. pneumoniae,* and *A. baumannii.* The oral anaerobes such as *P. gingivalis* are moving quickly to become members of this dangerous club. This trait makes the opportunists that share it particularly difficult to treat successfully. Even if a patient is eventually cured of the infection, delays engendered by having to find the right antibiotic can leave the patient with permanent damage.

The Dark Side of Some Residents of the Human Body

The Ever-Changing Face of *E. coli*

E. coli is a facultative anaerobe found in the environment as a common food contaminant responsible for food-borne illness and as a prevalent member of the intestinal microbiota of warm-blooded animals and humans. Most *E. coli* strains are nonpathogenic, and many that are part of the gut microbiota even benefit the host by providing vitamin K_2. However, other strains are associated with many different types of disease manifestations: diarrhea and dysentery in the gastrointestinal tract; bladder and kidney infections and **hemolytic uremic syndrome (HUS)** in the urinary tract; and systemic effects such as meningitis and septicemia. Habitat and high frequency of genetic exchange are the primary reasons *E. coli* strains have the ability to generate so many diverse forms of disease symptoms and outcomes. In general, different strains cause such a wide range of diseases because the strains have acquired different sets of virulence factors. We saw a similar pattern for Gram-positive opportunists in chapter 18.

Virotyping. Because of the high diversity found among *E. coli* isolates, initial attempts to classify *E. coli* strains were based on the traditional approach of **serotyping** using surface antigens: O-antigen of lipopolysaccharide (LPS) to determine the serogroup (~170 identified to date), flagellar H-antigen to determine the serotype (~55 identified to date), and capsular K-antigen to determine capsular type (used if the strain has a capsule). However, it soon became clear that many clinical isolates were not adequately distinguished by using this serotyping classification. It was found that many clinical strains often had the same serotype but very different virulence properties. Consequently, many researchers have switched to using **virotyping** as a means for classification, which is based on profiling the types of virulence factors present, including invasion and dissemination properties, adherence properties, toxins produced, effects on host cells, and symptoms produced. A summary of the assortment of virulence factors found in a few of the more commonly encountered clinical strains of *E. coli* associated with different diseases are shown in Figure 19-1. We will now consider each of these examples in more detail.

Enteroinvasive* E. coli *(EIEC). Strains of *Shigella* species (including ***Shigella dysenteriae,*** the causative agent of **dysentery**) and **enteroinvasive strains of *E. coli* (EIEC)** both cause symptoms of bloody diarrhea. If one looks just at their core "housekeeping" genes, then genetically they are nearly identical, and indeed many researchers have argued in favor of classifying *Shigella* as a virulent strain of *E. coli*; however, for historical reasons they are still named separately. In the early 1980s, a large (220 kb), highly homologous plasmid was found to be present in all virulent strains of *Shigella* and EIEC, which was responsible for the invasive phenotype observed for both bacteria. *Shigella* and EIEC are also able to spread from cell to cell through actin-based motility (actin-tail formation), similar to what is observed for *Listeria monocytogenes* (see Figure 11-14). Transfer of a wild-type invasion plasmid into avirulent, plasmid-cured *Shigella* strains or into *E. coli* strains lacking any plasmids reconstituted the invasive phenotype and converted the avirulent strains into virulent invasive strains. In addition to having invasive properties, *Shigella* strains produce **Shiga toxin (STx)**, which kills cells by depurinating (removing an adenine base from the ribose backbone) 28S rRNA at a specific position, thereby blocking protein synthesis (see Figure 12-11). STx action on intestinal cells damages the epithelium and underlying tissues, which results in the characteristic bloody diarrhea that is observed in dysentery. STx can cause acute irreversible kidney failure, known as **hemolytic uremic syndrome (HUS)**, particularly in children and the elderly. The *stx* gene encoding STx is located on a lambdoid bacteriophage integrated into the bacterial chromosome of *Shigella* species. EIEC strains do not produce STx and so do not cause HUS, but like *Shigella* they do invade cells and spread from cell to cell and cause intestinal tissue damage that results in diarrhea.

Enterotoxigenic* E. coli *(ETEC). A different situation occurs with **enterotoxigenic *E. coli* (ETEC)**, which is usually responsible for self-limiting, but sometimes severe, acute infant diarrhea. ETEC is the leading cause of so-called "traveler's diarrhea" encountered by tourists when visiting developing regions of the world. ETEC adhere to small intestinal mucosa via type 1 pili and type IV pili yet are noninvasive and cause little or no inflammation. However, they do produce one or both of two enterotoxins (see chapter 12). One is a small, peptide-derived **heat-stable toxin (HST)**, which is able to survive being boiled for up to 30 minutes, is resistant to acidic pH, and comes in two forms (STIa and STIb). The HSTs bind and activate guanylate cyclase receptors to increase intracellular levels of cyclic guanosine monophosphate (cGMP), resulting in activation of cGMP-dependent protein kinase (PKG), which in turn stimulates chloride ion channels and triggers diarrhea. The other enterotoxin is a larger, multisubunit, AB_5-type **heat-labile toxin (HLT)**, which comes in two forms (HLT-1 and HLT-2) and is related to cholera toxin (CT). Like CT, HLTs ADP-ribosylate and activate the α-subunit of the heterotrimeric Gs protein that regulates adenylyl cyclase to increase intracellular cAMP levels, resulting in activation of cAMP-dependent kinase (PKA) that leads to diarrhea (see Figure 12-20).

Enteropathogenic* E. coli *(EPEC). **Enteropathogenic strains of *E. coli* (EPEC)** also cause severe, acute infant diarrhea and "traveler's diarrhea," but the pathology is quite different from ETEC or EIEC. EPEC do not usually have genes for HST or HLT, but sometimes they do have other toxin genes, such as those for the **cytolethal distending toxin (CDT)**, which causes cell cycle arrest in G2 phase that leads to distension (enlargement) of host cells, and the **cytotoxic necrotizing factors (CNFs)**, which modify and activate small GTPases through deamidation or transglutamination to cause cytoskeletal rearrangements (see Table 12-1). The most notable characteristic of EPEC, however, is that they cause a phenomenon known as **enterocyte attachment and effacement (EAE)**, which causes an altered ultrastructure of the apical surface of mucosal epithelial cells. The bacterial attachment and interaction with the host cells results in a characteristic **pedestal formation**, which involves actin rearrangement and enlargement of short microvilli and elongation of the microvilli where the bacteria are bound. Concurrently, the microvilli disappear from areas where the bacteria are not bound. All EPEC genes necessary for the EAE phenotype are found on the chromosome within a 35.6-kb pathogenicity island termed the **locus of enterocyte effacement (LEE)**. The LEE contains genes encoding components of a type III secretion system (T3SS) apparatus (see chapter 13) and its secreted effector proteins (Esp effectors) that disrupt the host cell cytoskeleton, rearranging it to form the pedestals where the bacteria are attached. Another characteristic of EPEC strains is the production of **bundle-forming pili (Bfp)**, which are type IV pili similar to the toxin-coregulated pili (TCP) of *Vibrio cholerae* (see chapter 12) that are thought to be important for self-aggregation of bacteria (microcolony formation). The biosynthetic genes for Bfp are carried on a

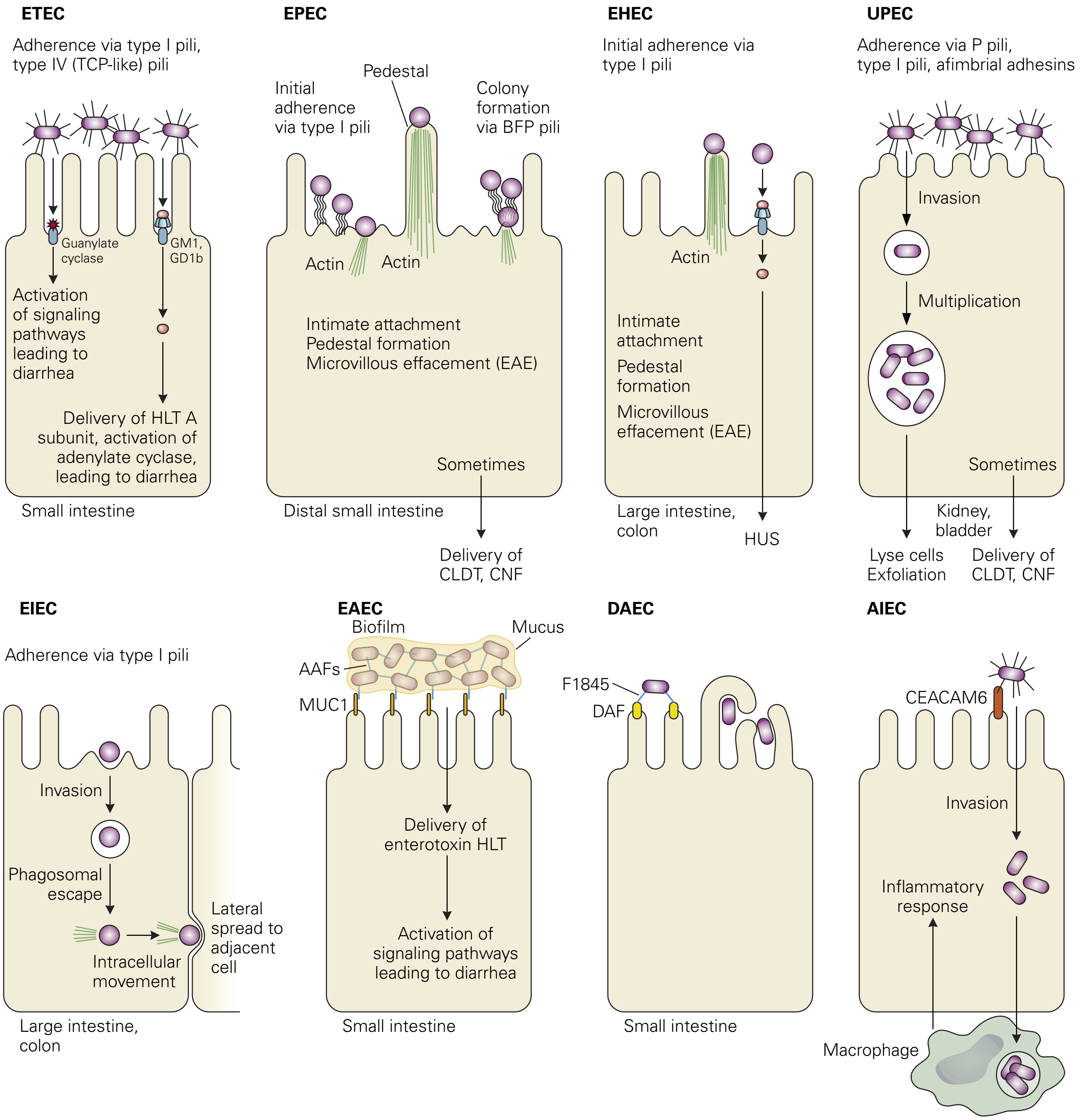
ETEC
Adherence via type I pili, type IV (TCP-like) pili
Guanylate cyclase
GM1, GD1b
Activation of signaling pathways leading to diarrhea
Delivery of HLT A subunit, activation of adenylate cyclase, leading to diarrhea
Small intestine
EPEC
Pedestal
Initial adherence via type I pili
Colony formation via BFP pili
Actin
Actin
Intimate attachment
Pedestal formation
Microvillous effacement (EAE)
Sometimes
Distal small intestine
Delivery of CLDT, CNF
EHEC
Initial adherence via type I pili
Actin
Intimate attachment
Pedestal formation
Microvillous effacement (EAE)
Large intestine, colon
HUS
UPEC
Adherence via P pili, type I pili, afimbrial adhesins
Invasion
Multiplication
Sometimes
Kidney, bladder
Lyse cells
Exfoliation
Delivery of CLDT, CNF
EIEC
Adherence via type I pili
Invasion
Phagosomal escape
Intracellular movement
Lateral spread to adjacent cell
Large intestine, colon
EAEC
Biofilm
Mucus
AAFs
MUC1
Delivery of enterotoxin HLT
Activation of signaling pathways leading to diarrhea
Small intestine
DAEC
F1845
DAF
Small intestine
AIEC
CEACAM6
Invasion
Inflammatory response
Macrophage

Figure 19-1. Examples of different virotypes of *E. coli*. *E. coli* that cause human disease are often classified into virotypes based on the complement of virulence factors that they display. Shown here are a select number of the more commonly encountered virotypes: enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), uropathogenic *E. coli* (UPEC), enteroinvasive *E. coli* (EIEC), *Shigella,* enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). However, new *E. coli* hybrids that have acquired unique combinations of the various virulence factors through multiple HGT events are emerging that muddle the picture and make discriminating among strains difficult. EAE, enterocyte attachment and effacement; TCP, toxin-coregulated pilus; CDT, cytolethal distending toxin; HST, heat-stable toxin; HLT, heat-labile toxin; CNF, cytotoxic necrotizing factor; BFP, bundle-forming pilus; HUS, hemolytic uremic syndrome; AAF, aggregative adherence fimbriae; CFA, colonization factor antigen; EAST1, EAEC Shiga toxin 1; ShET1, *Shigella* enterotoxin 1; DAF, decay accelerating factor; MUC1, epithelial transmembrane mucin 1. Based on information obtained from Nataro JP, Kaper J. 1998. *Clin Microbiol Rev* **11**:142–201; Kaper JB, Nataro JP, Mobley HL. 2004. *Nat Rev Microbiol* **2:**123–140; Kalita A, Hu J, Torres AG. 2014. *Curr Opin Infect Dis* **27**:459–464.

69-kb plasmid called the **EPEC adherence factor (EAF)** plasmid.

Enterohemorrhagic* E. coli *(EHEC), the Shiga Toxin-Producing* E. coli *(STEC). A recently emerged hybrid is **enterohemorrhagic *E. coli* (EHEC)**, also known as **Shiga toxin (STx)-producing *E. coli* (STEC)**. These strains are food-borne pathogens that cause bloody diarrhea and HUS like *Shigella*. EHEC/STEC strains have a mixed phenotype. They adhere to and efface intestinal epithelial cells (EAE phenotype) similar to EPEC but do not produce Bfp so as to aggregate like EPEC strains. They also cause HUS kidney disease like *Shigella* strains but do not invade or spread from cell to cell like *Shigella* or EIEC strains. EHEC/STEC strains produce several diarrheagenic enterotoxins, including **Shiga-like toxins (SLTs)** that are closely related to and have the same reaction mechanism as and cause HUS like STx from toxigenic *Shigella* strains (see chapter 12). The *slt* genes for SLT are located on lysogenic bacteriophage. Some strains carry the gene *stx1*, which encodes an SLT protein identical or nearly identical to that for the Shiga toxin from *Shigella*. Other strains carry *slt* gene variants, *stx2a* or *stx2b*, that encode antigenically distinct SLT proteins with only about 60% homology to the SLT-1 toxin.

EHEC/STEC first came on the scene in the early 1980s, when a now infamous strain, *E. coli* O157:H7, was isolated from several incidents of food poisoning associated with undercooked hamburger meat. But, it made its major public debut in 1993, when a large, multistate outbreak occurred involving 73 fast-food restaurants. There were a total of 732 cases, resulting in 171 hospitalizations, many of whom were children under 10 years of age suffering from serious kidney problems due to HUS (four children died). Since then, outbreaks of *E. coli* O157:H7 have been an all-too-common occurrence, and it is on the CDC's list of reportable diseases for epidemiological tracking. A new STEC strain, *E. coli* O104:H4, caused havoc thorough Europe in 2011. This strain is similar to O157:H7 strains in displaying a diarrheagenic HUS and EAE phenotype, but has a different O-antigen and capsule.

Uropathogenic* E. coli *(UPEC). Yet another virotype of *E. coli* are the **uropathogenic strains (UPEC)**, which constitute one of the most common causes of **urinary tract infections (UTIs)**, particularly in women. UPEC strains often produce hemolysins that can cause lysis of host cells. The unique feature of these strains is their propensity for colonization of the urinary tract (bladder) as opposed to the intestinal tract. UPEC strains produce type 1 and P pili that are critical for their ability to colonize the urinary tract (see chapter 11). Through the tip protein adhesin (FimH) of type 1 pili, UPEC strains bind to mannose-containing glycoproteins (uroplakins) located on the luminal surface of bladder epithelial cells and mediate invasion. In contrast, P pili bind specifically via a tip protein adhesin (PapG) to the disaccharide globobiose (α-D-Gal-1,4-β-D-Gal) linked to the sphingolipid ceramide (the P blood group antigen) that is found specifically on the membrane surface of host erythrocytes and uroepithelial cells. Although urine flow and a variety of host factors can impede contact of UPEC with superficial facet cells of the bladder, once contact is made, attachment and/or invasion can result, causing bladder infection (cystitis), leading to replication inside cellular vacuoles and eventually to lysis and **exfoliation** (sloughing) of the cells. Some of the bacteria end up infecting the underlying epithelial cells, where they can persist undetected by immune surveillance mechanisms. UPEC strains can also ascend further up the urinary tract and cause kidney infection (pyelonephritis).

Neonatal Meningitis-causing* E. coli *(NMEC). Two serious types of invasive infections that are on the rise, namely septicemia and **meningitis** (inflammation of the meninges, the membranes lining the brain and spinal cord), are caused by capsular type K1 strains of *E. coli*, called **neonatal meningitis-causing *E. coli* (NMEC).** Normally, most adults harbor K1 strains as benign commensals. However, if the colon wall is breached by surgery, rupture of the appendix, or other abdominal trauma, *E. coli* enters blood and tissue where it causes massive inflammation leading to septic shock. This is the primary form of *E. coli* sepsis seen in adults. In infants, however, an *E. coli* K1 strain infection is most commonly associated with sepsis and meningitis. In fact, next to group B streptococcal infections, NMEC is the second most common cause of neonatal meningitis (with each causing up to 35% of all cases). During birth, an infant can be exposed to *E. coli* in a variety of ways. The progression of neonatal meningitis is thought to begin with colonization of the gastrointestinal tract, followed by translocation across the intestinal mucosa. In most cases, this would not have serious consequences, but in the case of some *E. coli* strains, bacteremia develops, followed by invasion of the cerebral spinal fluid. In adults, the primary risk to survivors of delayed treatment of sepsis is damage to major organs caused by advanced sepsis. However, in the case of neonatal meningitis, the consequences of delayed treatment are more likely to be neurological complications such as cerebral palsy, learning disabilities, and hearing loss.

Given the different types of progression of *E. coli* invasive disease in adults and in neonates, it is not surprising that the major virulence factors of the causative strains are different. In adult cases, LPS appears to be the main virulence factor. Not all forms of *E. coli* LPS are equally dangerous. LPS molecules with 6 acyl groups on their lipid A molecule (hexa-acyl LPS) seem to be particularly effective at stimulating the endothelial cell response that results in massive cytokine release, triggering a sepsis cascade. An important feature of this type of LPS is that it is poorly recognized by the innate immune defenses that normally recognize and help eliminate commensal *E. coli* strains. Specifically, the TLR4 (Toll-like receptor) signaling response is not activated because the LPS-binding protein MD-2 does not bind to this modified LPS and so cannot form a signaling complex with TLR4 (see chapter 3).

In the case of *E. coli* K1 strains that cause neonatal meningitis, the K1 type of polysialic capsule seems to be the most important virulence factor, but there is considerable diversity and heterogeneity observed in the composite repertoire of virulence factors among NMEC strains. The K1 capsule itself confers serum resistance and can be variably O-acetylated, coating itself with sialic acid groups, thereby making itself poorly immunogenic because sialic acid is a molecule widely found on human cells. The **outer membrane protein A (OmpA)** of K1 strains can also interact with an endothelial cell glycoprotein (called Ecgp96) at the blood-brain interface and cause disruption of barrier integrity. Most NMEC strains also produce **cytotoxic necrotizing factor 1 (CNF1),** which disrupts barrier function by causing actin rearrangements in cells (see chapter 12). Finally, the inefficiency of the neonatal alternative complement cascade system, as well as a less developed adaptive immunity, hampers defense against encapsulated bacteria. For unclear reasons, but which may have something to do with their somewhat suppressed immune systems, pregnant women also appear to be at higher risk of colonization with the K1 capsular strains of *E. coli*. Colonization by the mother plays an important role in transmission to the neonate.

Other Hybrid* E. coli *Virotypes. As illustrated previously, by changing their complement of virulence factors, *E. coli* strains can dramatically change their virulence and pathological profiles. Horizontal gene transfer through plasmid conjugation and phage transduction has already generated a myriad of known phenotypes (see Figure 19-1), and these mixed strains exemplify the ease with which *E. coli* bacteria can convert from one virotype to another. Additional examples are included here. **Enteroaggregative *E. coli* (EAEC)** have several virulence plasmids that encode a variety of long **aggregative adherence fimbriae (AAF)** that bind to transmembrane mucins (such as MUC1) on intestinal epithelial cells, allowing bacteria to aggregate on the cell surface to form biofilms; however, the bacteria do not invade the host cells. EAEC plasmids also contain other virulence genes, encoding for a type VI secretion system (T6SS), the toxins hemolysin, enterotoxins and HSTs like ETEC or SLT like EHEC, and multiple antibiotic resistances. The **diffusely adherent *E. coli* (DAEC)** bind through a surface antigen (F1845) to the decay accelerating factor (DAF) on enterocytes to cause activation of signaling pathways leading to generation of long finger-like projections from host cells that wrap around the bacteria. **Adherent-invasive *E. coli* (AIEC)** can attach and invade through a macropinocytosis-like uptake process involving actin microfilaments and microtubules. The bacteria replicate inside large vacuoles in the intestinal epithelial cells and macrophages but do not spread from cell to cell like EIEC strains. They also do not cause HUS like EPEC, STEC, and *Shigella* or severe diarrhea like ETEC. Instead, they appear to trigger strong proinflammatory responses. Several lines of evidence suggest that AIEC may play a role in inflammatory bowel disease. AIEC are highly prevalent in patients with ileal-Crohn's disease and a mouse model of infection, where AIEC infection leads to chronic inflammation and fibrosis symptoms similar to those seen in patients with Crohn's disease. Clearly, the potential for new virotypes to emerge is high, and *E. coli* subgroups will appear that cause new variations of these diseases, depending on the combinations of virulence factor genes that they harbor.

Klebsiella pneumoniae Nosocomial Infections

Klebsiella are nonmotile, encapsulated, rod-shaped bacteria that are found ubiquitously in nature, including soil, water, plants, insects, animals, and humans. They often reside as part of the commensal microbiota of the nasal, oral, and gastrointestinal tracts of animals and humans, where the fecal-oral route is the most common mode of transmission. The two major virulence factors of *K. pneumoniae* that contribute to invasive disease are production of the iron-chelating siderophore **aerobactin** and the presence of a highly mucoid phenotype due to production of a capsule (encoding by the *cps* gene cluster), especially serotypes K1 or K2 that lack mannose groups normally recognized by the mannose-binding lectin (MBL)-mediated pathway of the complement cascade (see chapter 3). The capsule is also antiphagocytic and confers resistance to antimicrobial peptides such as β-defensins.

While the virulence of most nosocomial isolates of *K. pneumoniae* is not a significant problem for healthy individuals, in the immunocompromised, certain strains can wcause a variety of opportunistic nosocomial infections, including nosocomial pneumonia, neonatal infection, meningitis, diarrhea, UTIs, and septicemia. For more vulnerable populations such as hospital patients, acquisition of *K. pneumoniae* can have dire consequences for one major issue: the inability to treat the infections with antibiotics due to extensive drug resistance of most clinical isolates.

Hypervirulence. Over the past couple of decades, a new, highly virulent, invasive strain of *K. pneumoniae* has emerged, predominantly in the form of pyogenic liver abscesses that are not limited to immunocompromised individuals. The prominent feature of these community-acquired hypervirulent *K. pneumoniae* isolates is the pronounced **hypermucoviscous** phenotype (Figure 19-2), presumably due to more efficient iron acquisition and overproduction

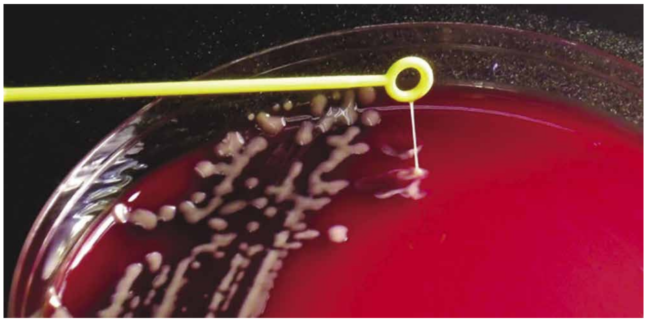

Figure 19-2. Hypermucoviscous phenotype of hypervirulent *K. pneumoniae*. Over production of capsule in the recently emerged hypervirulent strains of *K. pneumoniae* leads to the appearance of highly mucoid and viscous (i.e., hypermucoviscous) colonies. A diagnostic "string test" for this phenotype is considered positive if an inoculation loop or needle generates a viscous string >5 mm in length by lifting colonies straight up from an agar plate, as shown here. Reprinted from Shon AS, Bajwa RP, Russo TA. 2013. *Virulence* **4**:107–118, with permission.

of capsule. Comparative genome analysis has revealed that most hypervirulent clinical isolates belong to a single, recently evolved clonal lineage, called sequence type 23 (ST23) or clonal complex 23 (CC23). ST23 is distinct from nonhypermucoviscous, nonhypervirulent strains, such as ST258, due to acquisition of a single virulence plasmid and a chromosomal genomic island. The plasmid contains genes for two siderophores, **aerobactin** and **salmochelin**, and a regulator of the mucoid biosynthetic genes RmpA, while the genomic island contains genes for **yersiniabactin**, a siderophore found in *Yersinia* species; **colibactin**, a **genotoxin** that causes double-stranded DNA breaks and senescence in mammalian cells; and **microcin E492**, a membrane channel-forming bacteriocin that is active against *E. coli* and also causes apoptosis in mammalian cells.

Multidrug Resistance. Multidrug resistant strains of *K. pneumoniae* have emerged as some of the most-feared pathogens in the hospital setting, the so-called ESKAPE pathogens, where most clinical isolates display a formidable array of drug resistance mechanisms (see chapter 16). These nosocomial **multidrug resistant (MDR)** strains, including the predominant pandemic clonal nonhypervirulent strain of sequence type 258 (ST258), also called clonal complex 258 (CC258), carry a number of chromosomal pathogenicity islands and transmissible plasmids with multiple genes encoding multidrug efflux pumps, extended-spectrum β-lactamases (ESBLs), carbapenemases, aminoglycosides, trimethoprim/sulfonamides, chloramphenicol, tetracyclines, and fluoroquinolones. Recently, there has also been an alarming spread of transmissible plasmid-mediated resistance to some of the drugs of last resort, such as colistin and tigecycline, which are normally used to treat infections of MDR *K. pneumoniae*. Given the relatively high frequency of horizontal gene transfer (HGT) involving antibiotic resistance-conferring plasmids among *K. pneumoniae*, there is mounting fear that MDR isolates of hypervirulent strains will soon appear on the health scene.

Because of the urgent need to find treatment options for MDR *K. pneumoniae*, researchers have begun to turn to alternative strategies to get around the antibiotic resistance problem. One exciting strategy that is gaining momentum is the use of *K. pneumoniae*-specific lytic bacteriophage, such as the newly discovered ϕBO1E that targets only carbapenemase-producing *K. pneumoniae* isolates belonging to the major pandemic clonal group (ST258). The thought is that the phage might even be used as a preventive measure to selectively decolonize patients that have *K. pneumoniae* as part of their commensal microbiota.

Bacteroides fragilis—The Bad Sheep of the Family

The genus *Bacteroides* is one of the major groups of bacteria in the colonic microbiota, comprising nearly half of the gut microbiota, with the others being the Gram-positive obligate anaerobes belonging to the phyla Firmicutes (such as Clostridia and Lactobacilli) and Actinobacteria and the Gram-negative obligate or facultative anaerobes of the phylum Proteobacteria. *Bacteroides* are Gram-negative, obligately anaerobic, rod-shaped bacteria. Some members of the *Bacteroides*, such as *Bacteroides thetaiotaomicron*, are critical for host nutrition and development of mucosal immunity. Although *B. fragilis* is not present in numbers as high as other *Bacteroides* species, it is the one most commonly associated with *Bacteroides* infections.

***Enterotoxigenic* B. fragilis.** A subset of *B. fragilis* strains (enterotoxigenic *B. fragilis*) causes diarrheal disease and colitis in animals and humans. These strains produce one of three subtypes of a 45-kDa proenzyme **enterotoxin (BFT or fragilysin)**, BFT1 through BFT3. BFT requires processing for activation by another *B. fragilis*-secreted cysteine protease called **fragipain (Fpn)** to remove a 20-kDa autoinhibitory domain. Activated BFT has zinc-dependent metalloprotease activity that binds to and activates receptors on colonic epithelial cells (CECs) and causes cleavage of E-cadherin to disrupt adherens junctions, activates β-catenin and Wnt signaling pathways, and induces IL-8 release from CECs, which in turn results in diarrhea, persistent mucosal production of IL-17, and persistent colitis (Figure 19-3A). Long-term colonization with enterotoxigenic *B. fragilis* is associated with increased risk of colorectal cancer, which is supported by mouse infection models that demonstrate accelerated tumorigenesis after as little as 3 months (Figure 19-3B).

***Nonenterotoxigenic* B. fragilis.** Non-BFT-producing strains of *B. fragilis* (NTBF) are responsible for causing the main types of infection associated with *Bacteroides*, namely intra-

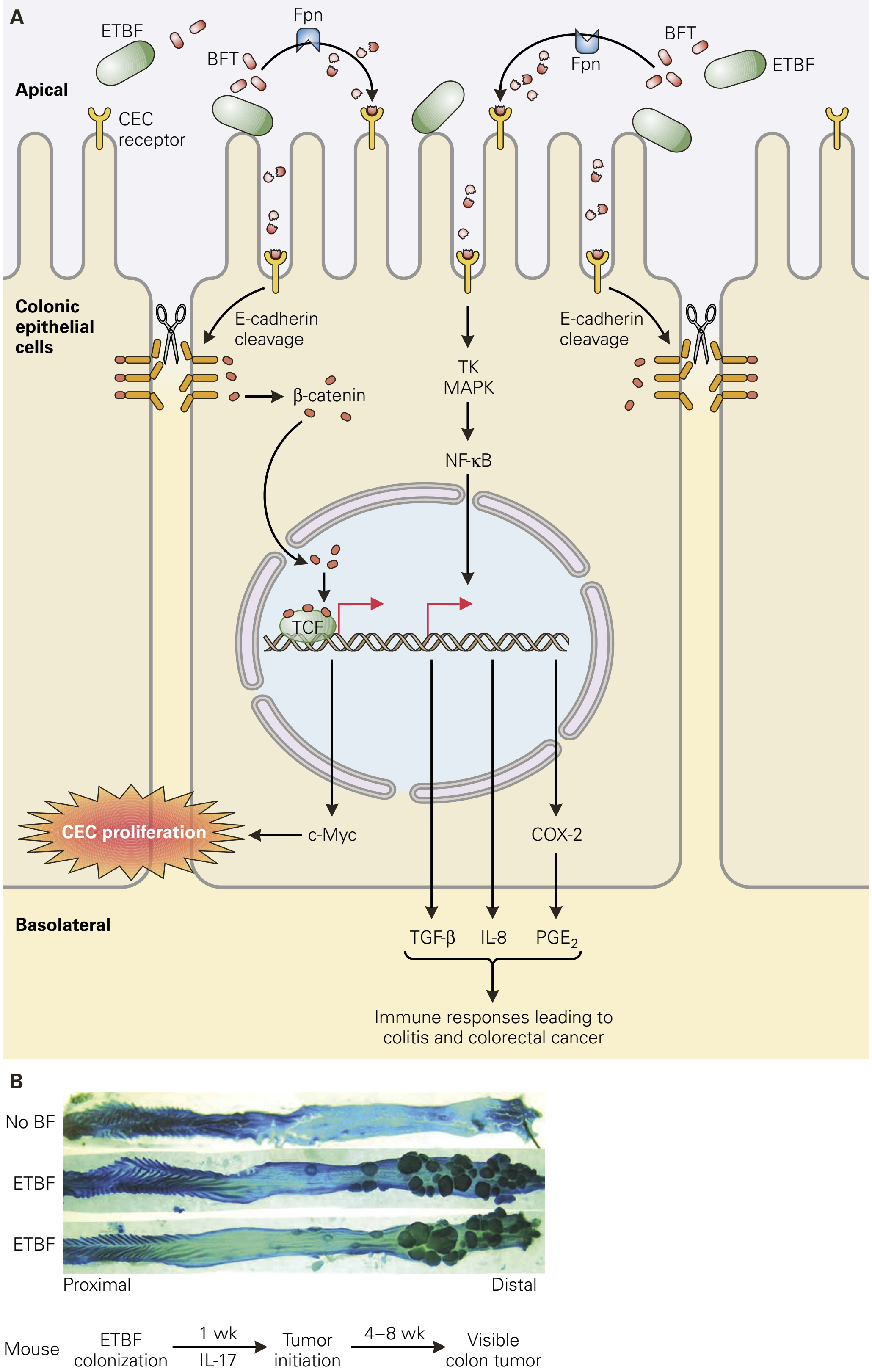
A
ETBF
Fpn
BFT
BFT
Fpn
ETBF
Apical
CEC
receptor
Colonic
epithelial
cells
E-cadherin
cleavage
β-catenin
TK
MAPK
NF-κB
E-cadherin
cleavage
TCF
CEC proliferation
c-Myc
COX-2
Basolateral
TGF-β
IL-8
PGE2
Immune responses leading to
colitis and colorectal cancer
B
No BF
ETBF
ETBF
Proximal
Distal
Mouse
ETBF
colonization
1 wk
IL-17
Tumor
initiation
4–8 wk
Visible
colon tumor

Figure 19-3. Model of enterotoxigenic *Bacteroides fragilis*-mediated colitis and tumorigenesis. (A) Model of enterotoxigenic *Bacteroides fragilis* (ETBF)-mediated colitis. ETBF strains secrete one of three subtypes of a zinc-dependent metalloprotease toxin (BFT), which is a proenzyme that gets activated by another secreted cysteine protease called fragipain (Fpn) into a mature 20-kDa protein. Activated BFT binds to receptors on intestinal epithelial cells and elicits disruption of adherens junctions through presenilin-1 (PS1)-mediated cleavage of E-cadherin, activation of β-catenin and Wnt signaling that leads to expression of c-Myc, which promotes colonic epithelial cell (CEC) proliferation, and the pro-inflammatory cytokines transforming growth factor β (TGF-β), IL-8, and prostaglandin PGE_2 (via COX-2), which stimulate immune responses leading to colitis and colorectal cancer. TCF, T cell transcription factor; TK, tyrosine kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor kappa enhancer of activated B cells. Adapted from Sears CL. 2009. *Clin Microbiol Rev* **22**:349–369, with permission. **(B)** ETBF-mediated colon tumorigenesis in a mouse model of infection. ETBF induces mucosal IL-17 production 1 week after inoculation of mice, which persists, and marked tumor formation in the distal colon by 3 months. Taken from Sears CL, Geis AL, Housseau F. *J. Clin Invest* **124**:4166–4172 with permission.

abdominal abscesses and bacteremia. Like *E. coli* disease in adults, the cause of *B. fragilis* infections is trauma to the abdominal area, primarily from wounds or during surgery, that leads to breaches in the colon wall that allow the bacteria to enter tissue and blood. NTBF infections could be considered an emerging disease in the sense that such infections were unknown until an effective antibiotic therapy against invasive *E. coli* infections was developed. *E. coli* septicemia kills so much more quickly that it tended to obscure the much more slowly developing *B. fragilis* infection. The antibiotic mixture that effectively prevented most cases of *E. coli* septicemia was a mixture of an aminoglycoside and a β-lactam antibiotic. *B. fragilis* strains proved to be resistant to this combination.

Another reason *B. fragilis* infections were not formally recognized until the 1970s was that most physicians believed that obligate anaerobes could not cause infection in what they viewed as the aerobic milieu of human tissue and blood. What they failed to take into account is that regions of dead tissue become highly anoxic due to loss of their blood supply. Such lesions are present in most adults and can occur in any organ of the body, including the lungs. NTBF is able to colonize such dead tissue and form abscesses that can leak bacteria into the bloodstream. Since most of the oxygen in blood is not free, but instead is bound by hemoglobin, a relatively oxygen-tolerant bacterium like *B. fragilis,* which cannot grow in the laboratory if oxygen is present but can survive oxygen exposure for long periods, survives in blood well enough to cause bacteremia.

Some progress has been made toward understanding how *B. fragilis* causes disease. As has already been mentioned, the ability of *B. fragilis* to survive in the human body is due in part to its ability to colonize regions of damaged tissue. Production of polysaccharidases and proteases probably contributes to its ability to evade the immune response and grow and disseminate in tissue. The major virulence factor of NTBF is its unusual, complex polysaccharide capsules. NTBF displays 8 distinct polysaccharides A through H (PSA through PSH), each of which can undergo phase variation via inversions of promoters upstream of 7 of the 8 cognate polysaccharide biosynthesis gene operons. These PS capsules have been shown to be important for adherence and abscess formation in animals, even when no bacteria are present. Moreover, unlike most carbohydrate antigens, which are T cell independent, PSA stimulates T cell activity, restoring the balance of Th1 and Th2 responses and contributing to healthy mucosal immunity. Indeed, PSA alone has been shown to protect against development of colitis.

A clinically important feature of *B. fragilis* and other *Bacteroides* species that can cause opportunistic human infections is their increasing resistance to a variety of antibiotics. They are now almost universally resistant to all tetracyclines. The macrolide antibiotics (clindamycin and erythromycin) that have been used to treat them are losing their efficacy due to the horizontal spread of genes conferring resistance to these antibiotics (see chapter 16). Rapid spread of these resistance genes can actively occur in the colon via integrated mobile elements called conjugative transposons, preparing the bacteria to resist treatment should an infection occur.

Porphyromonas gingivalis—A Keystone Pathogen

P. gingivalis is related to *Bacteroides* species (both belong to the order Bacteroidales), but whereas the main location of *Bacteroides* species is in the colon, *P. gingivalis* is located in the mouth. Like *B. fragilis,* the black-pigmented *P. gingivalis* is an obligate anaerobe. You might think at first that the mouth is an aerobic environment, and it is for bacteria that stick to the exposed surface of teeth or on the tissue of the mouth lining. However, the periodontal pocket, the region between the teeth and gums (the gingiva), is quite anoxic and this is where *P. gingivalis* resides. *P. gingivalis* has multiple ways of evading host immune responses, including fimbriae that bind to host cell surface integrins and help in biofilm formation; a capsular polysaccharide that plays a role in abscess formation; and a number of immunomodulatory proteases with multiple functions in modulating immune responses, enhancing in vivo survival, and contributing to tissue damage and dissemination in the body.

Periodontal Disease. *P. gingivalis* is, as the name suggests, involved in the inflammatory gum disease gingivitis (see Figures 6-9 and 6-10). As we discussed in chapter 6, *P. gingivalis* is a **keystone pathogen** that is normally present in low numbers in the oral microbiota but can have a disproportionate impact on the microbial community, eventually leading to a shift in the microbial composition. It is important to realize that *P. gingivalis* is not the only Gram-negative anaerobe involved in periodontal disease. A number of other unrelated species (*Tannerella forsythia, Fusobacterium nucleatum, Treponema denticola, Aggregatibacter actinomycetemcomitans, Prevotella intermedia,* and *Eikenella corrodens*) are members of the oral microbial community that have also been associated with periodontal disease. *P. gingivalis*, along with other Gram-negatives, exploits the resulting dysbiotic conditions to grow. The result is inflammation, which causes a deepening of the periodontal pocket, bleeding gums, resorption of bone, and even progression to tooth loss. In fact, periodontal disease is the main cause of tooth loss in adults, where it is mainly associated with poor oral hygiene, but there is a form of periodontal disease, called juvenile periodontitis, that affects younger people and aggressively leads to disease and early tooth loss. There is probably also a human genetic factor because some populations have a higher incidence of periodontal disease than others.

P. gingivalis has been intensively studied with respect to its virulence factors. The two major virulence factors that appear to be important for the type of tissue damage that occurs during periodontal disease are: fimbriae, which mediate attachment to the tooth surface below the gum, and proteases that damage tissue and alter immune responses. Enzymes such as chondroitin sulfatase and hyaluronidase, which degrade host tissue polysaccharides, may also make a contribution to tissue destruction. *P. gingivalis* secretes two major proteases: Arg-specific gingipain (Rgp), encoded by the *rgpA* and *rgpB* genes, and Lys-specific gingipain (Kgp), encoded by the *kgp* gene. In addition to the N-terminal catalytic protease domain, RgpA and Kgp also have a C-terminal hemagglutinin/adhesin (HA) domain; RgpB lacks this HA domain. These cysteine proteases not only damage tissue directly and provide food and Fe-bound hemin for the bacteria, but they can degrade immune molecules, such as antibodies and complement components. Kgp cleaves IgG1 and IgG3 and thereby impairs bacteria opsonization by neutrophils. Kgp also cleaves C5aR. Rgp cleaves C3 into C3a and C3b; C3a induces pro-inflammatory cytokines. Rgp-mediated cleavage of the C5 protein into C5a and C5b releases C5a, a chemoattractant for neutrophils, which may explain the massive influx of neutrophils that occurs during gingivitis and the ensuing alteration of the microbiota that leads to periodontal disease (see Figure 6-10).

Most cases of periodontal disease are still treated by surgery that peels back the gum tissue so that plaque can be scraped from the roots of the teeth. This type of treatment is not pleasant and is very expensive, and not surprisingly, new laser-based surgery techniques are starting to gain popularity. There is also hope that antibiotics, particularly in multidrug combinations, might be used to prevent or even treat periodontal disease. A problem with using antibiotic treatment, however, is that the bacteria form biofilms on the tooth surface, and biofilm-embedded bacteria are generally more resistant to killing by antibiotics than planktonic bacteria.

Oral Bacteria and Coronary Artery Disease. *P. gingivalis* can occasionally cause internal abscesses, but the most serious internal condition associated with invasive *P. gingivalis* is cardiovascular disease. Mounting evidence suggests that *P. gingivalis* does not merely colonize atherosclerotic plaques in blood vessels, but it is now widely accepted that *P. gingivalis* actually contributes to causing cardiovascular disease. This finding opens up the possibility that antibiotics could be used to prevent or treat some forms of heart disease.

Once *P. gingivalis* binds via its adhesins, such as fimbriae FimA, to cellular receptors (β integrins CD11b/CD182, CD14, and ICAM-1), it can invade human coronary artery endothelial cells through receptor-mediated endocytosis (Figure 19-4). The bacteria are then trafficked through the autophagy pathway (see chapter 3), a process that normally allows the cell to degrade damaged or surplus organelles such as mitochondria. But, in the case of *P. gingivalis,* the bacteria-containing phagosome evades phagolysosomal fusion and instead trafficks to the early autophagosome, where they fuse. Once the bacteria are in the late autophagosome, fusion with the lysosome is again blocked and the bacteria reside in the specialized vacuole, where they are able to replicate and even persist for some time. At some point, the bacteria exit the cells through the endocytic recycling pathway to infect other nearby cells.

It should be noted that not all *P. gingivalis* survive once taken up by host cells, since there is some trafficking that occurs to the lysosome. There has also been considerable variation observed among clinical isolates of *P. gingivalis* in terms of their invasiveness and persistence properties. And, of course, there is host variation to consider with regard to susceptibility to periodontal disease or coronary artery disease associated with this oral opportunist. Another potential indirect involvement of oral anaerobes in adverse health outcomes, this time *F. nucleatum,* is described in Box 19-1.

Environmental Inhabitants Weigh in as Opportunists

Pseudomonas aeruginosa—A Versatile Opportunist of the Highest Order

P. aeruginosa has been called the consummate opportunist. *P. aeruginosa* is a soil microorganism with an impressive capacity to utilize a variety of carbon and energy sources. As a facultative anaerobe that has multiple iron-acquisition mechanisms, *P. aeruginosa* easily adapts from aerobic to microaerobic to anaerobic conditions with limited nutrient sources. Due to its versatility in carbon source utilization, *P. aeruginosa* has been widely used for bioremediation of such toxic compounds as toluene and tricloroacetate. Unfortunately, *P. aeruginosa* is also capable of causing serious disease in humans. *P. aeruginosa* can cause a variety of infections (see Table 19-1), ranging from bloodstream infections in immunocompromised patients to UTIs and hot tub folliculitis (inflammation of hair follicles from colonization with *P. aeruginosa* acquired from hot tubs and other warm aquatic places). In addition, *P. aeruginosa* is a leading cause of contact-lens-induced keratitis and corneal ulcers caused by misuse of contact lenses, even in healthy individuals. But *P. aeruginosa* is best known for its ability to infect damaged, exposed tissue in wound and burn victims and to cause lung infections in people with **cystic fibrosis (CF)** and in hospital patients with ventilator-associated pneumonia.

Wound and Burn Infections. The predilection of *P. aeruginosa* for damaged wound or burned tissue is easy to understand since it is likely to be on the scene due to its ubiquitous presence in the environment. Wounds provide access to blood and tissues and burned tissues seep nutrient-rich

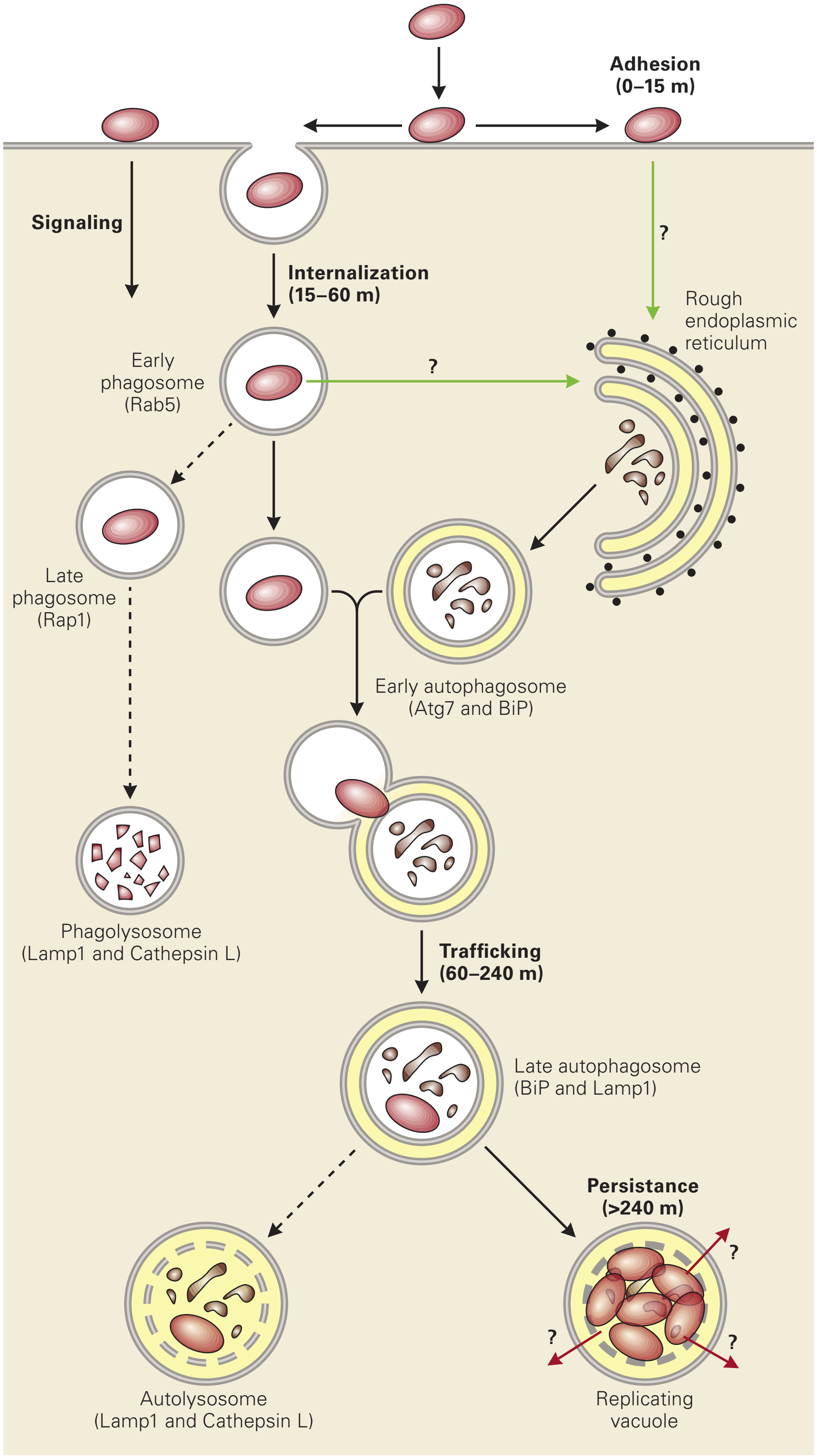
Adhesion
(0–15 m)
Signaling
?
Internalization
(15–60 m)
Rough
endoplasmic
reticulum
Early
phagosome
(Rab5)
?
Late
phagosome
(Rap1)
Early autophagosome
(Atg7 and BiP)
Phagolysosome
(Lamp1 and Cathepsin L)
Trafficking
(60–240 m)
Late autophagosome
(BiP and Lamp1)
Persistance
(>240 m)
?
?
?
Autolysosome
(Lamp1 and Cathepsin L)
Replicating
vacuole

Figure 19-4. Model for *Porphyromonas gingivalis* invasion and persistence. Most strains of *Porphyromonas gingivalis* are able to invade and reside within host vascular cells and tissue, including cardiovascular cells and cells within atherosclerotic plaques. The invasion process involves bacterial fimbrial attachment to β-integrin (CD11b/CD182), CD14, or ICAM-1 receptors on host cells, such as coronary artery endothelial cells, followed by receptor-mediated endocytosis. Once in the endosome (Rab5 marker), the fusion of the late phagosome (Rap1 marker) with lysosome (Lamp1 and cathepsin L markers) to form the phagolysosome is blocked, and instead the bacteria-containing vacuole is trafficked to the early autophagosome (Atg7 and BiP markers). Fusion of the late autophagosome (Lamp1 and BiP markers) with lysosomes is also blocked, and instead the bacteria reside and replicate within the specialized vacuole. At some point, the bacteria are able to exit the cell again through the endocytic recycling pathway so as to invade another cell. Adapted from Olsen I, Progulske-Fox A. 2015. *J Oral Microbiol.* **7**:28788, with permission.

Box 19-1.
Fusobacterium nucleatum—A Cause of Preterm Birth?

Preterm birth, defined as birth before 37 weeks of gestation, is well known as a condition that is associated with developmental defects and other lifelong consequences. There are undoubtedly many causes of preterm birth, but now an unexpected one has surfaced—gum disease. Oral anaerobes, such as *Fusobacterium nucleatum,* a species with a proven capacity to cause inflammation and tissue damage, may be involved in a wide range of diseases, including periodontitis, oral cancer, and preterm birth. *F. nucleatum* has been isolated from placentas and uterine fluid of a number of women who experienced preterm birth. But this was still just guilt by association. The bacteria could have been enabled to infect by some other condition. In mouse models, *F. nucleatum* has been clearly shown to cause preterm birth. Granted, animal models can be misleading, but this advance is a step toward making a cause and effect connection. Support of this connection comes from the prevalence of the bacterium in extra-oral sites of infection and the fact that antibiotic intervention prevents preterm birth in cases of infection. Additionally, the FadA adhesin/invasin is a conserved virulence factor that plays a role in its ability to cause infection.

Preterm birth has also been associated with bacterial vaginosis, a shift in the vaginal microbiota from predominantly Gram-positive to predominantly Gram-negative bacteria. In this case, antibiotic intervention that restored the Gram-positive dominance in the vaginal tract has a protective effect. Although the view of what factors influence preterm birth is still murky and although it seems clear that there are multiple possible causes of preterm birth, it is likely that at least some of the predisposing factors are bacterial.

Sources:

Liu H, Redline RW, Han YW. 2007. *Fusobacterium nucleatum* induces fetal death in mice via stimulation of TLR4-mediated placental inflammatory response. *J Immunol* **179:**2501–2508.

Han YW. 2015. *Fusobacterium nucleatum:* a commensal-turned pathogen. *Curr Opin Microbiol* **23:**141–147.

plasma, both of which the bacteria can quickly take advantage of to proliferate. The bacteria also have a notorious predilection for adhering, aggregating, and forming biofilms on all kinds of surfaces. *P. aeruginosa* infections are the most likely cause of death of patients who have survived the initial trauma of severe burns, so physicians are ever vigilant for early signs of *P. aeruginosa* infections in these patients. It is essential to treat *P. aeruginosa* infections as early as possible for two important reasons. First, *P. aeruginosa* is well known for its intrinsic and HGT-acquired resistance to a variety of antibiotics. Biofilm formation on the damaged tissue makes it even more resistant to treatment with antibiotics. Second, what actually ends up killing the patient is when the bacteria get into the bloodstream and cause sepsis. So it is important to treat the infection early and effectively in order to prevent movement of bacteria into the bloodstream. However, the greatest challenge is combatting infections with MDR strains of *P. aeruginosa*, which are pervasive nowadays in hospital settings. Other bacteria, such as *Staphylococcus aureus* (see chapter 18), also cause life-threatening wound and burn infections, but *P. aeruginosa* is the leading cause of such infections.

Lung Infections in Cystic Fibrosis Patients. *P. aeruginosa* causes particularly problematic infections in the lungs of CF patients. CF is an inherited chronic lung disease caused by a mutation in the **cystic fibrosis transmembrane conductance regulator (CFTR)**, a cAMP-regulated chloride ion (Cl^-) channel (see Figure 12-20). The mutation causes misfolding of the protein that leads to unregulated Cl^- (and consequently Na^+) ion efflux. The resulting water adsorption causes, among other things, dehydration of the airway surface and a thickening of the mucus layer in the lungs and airways. This thick mucus obstructs the airway and inhibits ciliary movement, causing coughing and wheezing. Interference with the ciliated cell defenses of the airway that propels bacteria trapped in mucus out of the airway makes it easier for bacteria to colonize and form robust and enduring biofilms. An important contributor to early tissue damage in the CF lung appears to be release of **neutrophil extracellular traps (NETs)**, a sticky matrix of DNA-histone fibers with proteases, elastases, cathepsin, and myeloperoxidase (a generator of reactive oxygen species) that is released into the extracellular space in response to interaction with bacteria (Figure 19-5). The biofilms cause further **ciliary dyskinesia** (inability of ciliated cells to move and remove mucus from the lungs) and chronic infection that can eventually lead to early death of the CF patient.

Within the first few years of life, most CF patients acquire *P. aeruginosa* in their lungs. Oddly enough, although most CF patients are colonized early in life, lung damage and reduced lung capacity do not occur abruptly. Rather the effects of the infection develop slowly over a period that can extend into many years. Treatment of the infection with

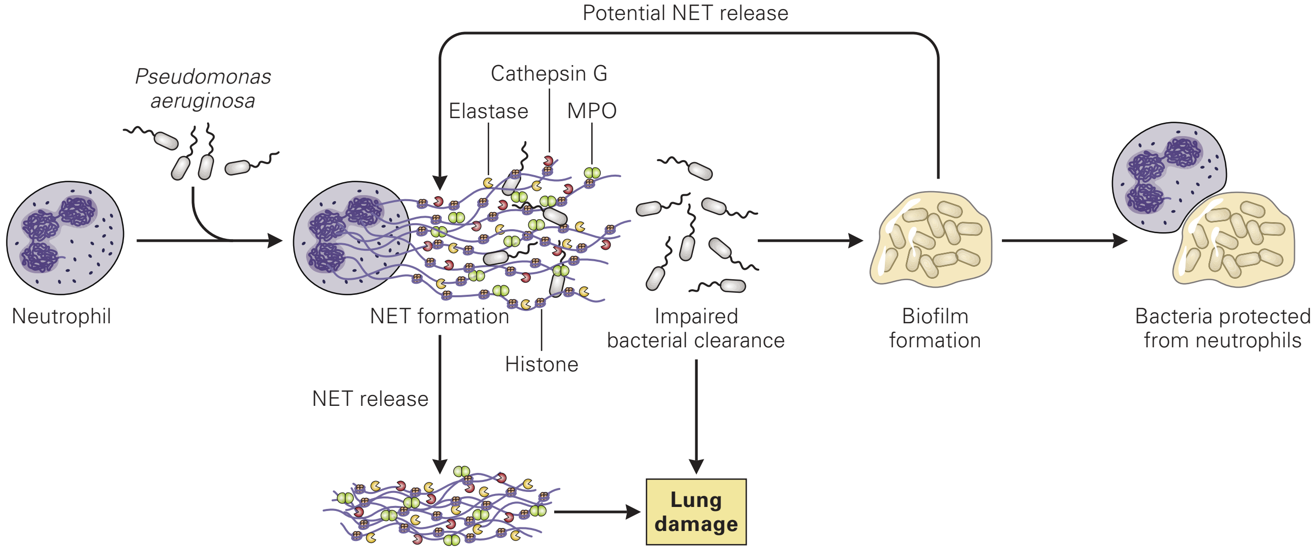

Figure 19-5. The interplay of *Pseudomonas aeruginosa* and neutrophils in cystic fibrosis lung infections. In a cystic fibrosis patient, impairment of cilia function facilitates colonization of lung by opportunists such as *Pseudomonas aeruginosa.* Early interactions of the bacteria with lung neutrophils lead to neutrophil extracellular trap (NET) formation, which further impairs bacterial clearance, contributes to lung damage, and promotes biofilm formation. Bacteria within the biofilms are protected from phagocytic killing by neutrophils, yet NET release continues to cause damage to the lung tissues. Based on information from Rada B. 2017. *Pathogens.* **6**:10, with permission.

antibiotics slows the progression of the damage, but it does not clear the bacteria from the lung. Gradually, changes in physiological traits of the bacteria, such as production of an alginate capsule, and increasing resistance to antibiotics (particularly as a result of constant selective pressure due to repeated antibiotic administration) combine to impair the lungs to a great enough extent that the patient dies. At one time, CF patients invariably died early in life, but improvements in antibiotic treatment and management of the associated chronic infections have now extended the lifespan of CF patients up to the 40s and 50s. Thus, although the problem of *P. aeruginosa* infections in CF patients has not been solved, great improvements have been made. Indeed, widespread efforts over the past couple of decades to eradicate initial acquisition of *P. aeruginosa* in childhood have greatly reduced the prevalence of *P. aeruginosa* in CF patients (Figure 19-6A). Unfortunately, with the exit of one opportunist, in comes another that is ready to take its place—only in this case, it's more than one: *S. aureus*, *Stenotrophomonas maltophilia* (formerly *Xanthomonas maltophilia*), *Haemophilus influenzae*, *Burkholderia cepacia* complex (BCC), and *Achromobacter* species (Figure 19-6B).

Virulence Factors. The virulence factors of *P. aeruginosa* involved in colonization, survival in the external and host environments, tissue damage, and septicemia are summarized in Table 19-2. The bacteria have a single polar flagellum for motility that responds to chemotaxis. The bacterial cell surface displays multiple pili and afimbrial adhesins. There are also a number of secreted tissue damaging toxins and enzymes, such as proteases, hemolysins, exotoxin (ExoA), toxic T3SS effector proteins (ExoS, ExoT, ExoU, and ExoY), and the nonprotein redox-active toxic pigment, **pyocyanin** (see chapter 12). The proteases, hemolysins, and phospholipases have relatively nonspecific activities that damage the extracellular matrix of connective tissue and surfaces and membranes of eukaryotic cells.

Exotoxin A (ExoA) is a toxin secreted by T2SS that binds mammalian cells, enters via receptor-mediated endocytosis, and translocates the toxic catalytic domain into the cytoplasm, where it then ADP-ribosylates elongation factor-2, an essential factor in protein synthesis (see chapter 12). Exotoxins S (ExoS) and T (ExoT) are injected directly into the mammalian cell via a T3SS (see chapter 13). These toxins are both bifunctional enzymes, with a GTPase-activating activity domain and ADP-ribosyl transferase activity domain. Although these two toxins act against different intracellular targets, they both result in the same cellular outcome—to disrupt the mammalian cell cytoskeleton and trigger an apoptosis-like cell death. Exotoxins U (ExoU, a phospholipase) and Y (ExoY, an adenylyl cyclase of unknown function) are also injected by the T3SS. Recent studies have revealed insights into the roles that some of these virulence factors play in dissemination from the lung into the bloodstream during infections by pathogenic strains of *P. aeruginosa* (Figure 19-7).

A different type of cytotoxin is the pigment pyocyanin (see Figure 9-2), which gives colonies of *P. aeruginosa*

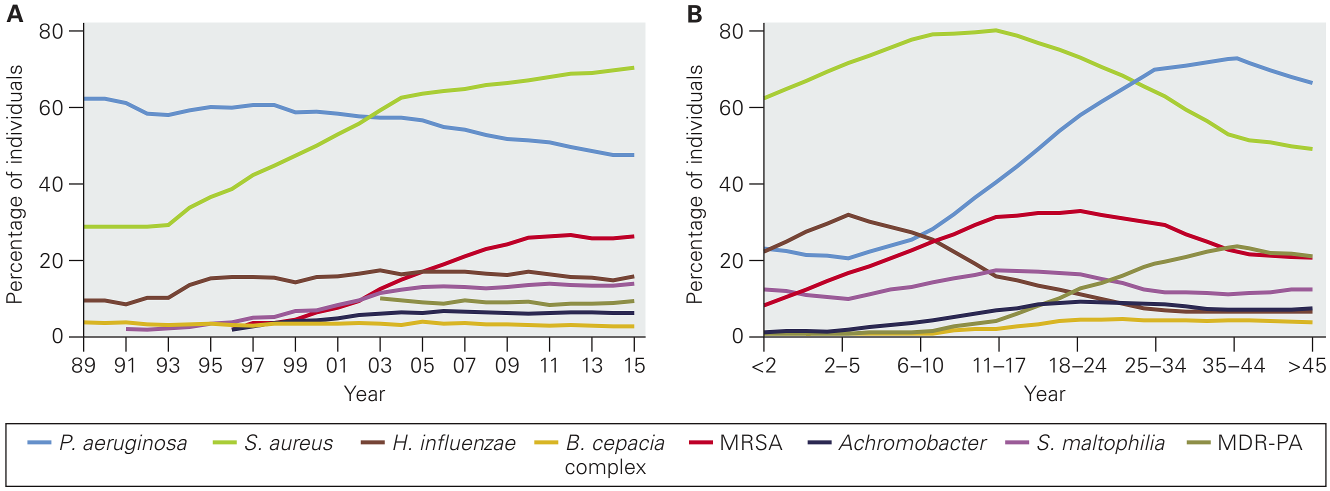

Figure 19-6. Prevalence of respiratory bacteria in the lungs of cystic fibrosis patients. Cystic fibrosis (CF) patients suffer lifelong pulmonary problems from chronic infections with opportunistic pathogens, as well as with outright pathogens. The most common respiratory bacteria found in lungs of CF patients include *Pseudomonas aeruginosa, Staphylococcus aureus, Haemophilus influenzae, Stenotrophomonas maltophilia, Achromobacter* species, and *Burkholderia cepacia complex* (comprised of *B. cepacia*, *B. multivorans*, *B. gladioli*, *B. cenocepacia*, and other *Burkholderia* species). **(A)** Prevalence of bacteria found in CF lungs reported over the period from 1989–2015. **(B)** Prevalence of bacteria found in CF lungs according to age group. MRSA, methicillin-resistant *S. aureus*; MDR-PA, multidrug resistant *P. aeruginosa*. Data from Cystic Fibrosis Foundation. Cystic Fibrosis Patient Registry: Annual Data Report, 2015. https://www.cff.org/Our-Research/CF-Patient-Registry/2015-Patient-Registry-Annual-Data-Report.pdf

their characteristic blue-green color on agar medium. Large amounts of pyocyanin are produced in the lungs of CF patients. Pyocyanin interferes with the normal redox cycle in mammalian cells, leading to production of reactive forms of oxygen that damage the mammalian cell. Pyocyanin may also be a factor in tissue necrosis that occurs during infections of burned areas. The production of the blue pigment by the bacteria is seen in burn patients as blue pus. This pigment fluoresces under long wavelength UV light. In fact, shining a Woods lamp on a burned area to detect this fluorescence is an old but useful way to detect early signs of a *P. aeruginosa* infection in burn victims.

Another nonprotein toxin of *P. aeruginosa* is LPS. As for other Gram-negative pathogens, LPS activates TLR4-mediated cytokine release and the alternative complement cascade. Still other nonprotein toxins produced by *P. aeruginosa* are **rhamnolipids**, which form a barrier or shield around the bacteria. These lipids are toxic for phagocytic cells, such as neutrophils, and so protect the bacteria from the innate immune system. These rhamnolipids also modulate colony morphology and motility of other bacteria, especially in biofilms. For instance, in the CF lung, high concentrations of *P. aeruginosa*-derived rhamnolipids will kill species of the BCC (see next section), but low levels of the toxins can change colony morphology and reduce surface tension so as to induce swarming motility. Finally, quorum sensing, in the form of the three, interconnected cell-to-cell signaling systems (Las, Rhl, PQS), controls expression of many virulence factors and aids in the formation of biofilms.

Thus, *P. aeruginosa* has a highly complex repertoire of activities that allow it to be a successful pathogen in certain compromised hosts, particularly when the outer defenses have been breached. While we are beginning to understand the role of these factors in the special ability of *P. aeruginosa* to colonize and damage the lungs of CF and other immunocompromised patients, the sequence of action of these factors during the long progressive development of infection is still under study. Another very interesting question is what the *P. aeruginosa* virulence factors and toxins do when the bacteria are out in the environment or interacting with other hosts, such as amoeba.

Despite the T3SS and its effector proteins playing a major role in *P. aeruginosa* virulence, high diversity is observed among clinical isolates. Indeed, recently new virulent strains of *P. aeruginosa* have emerged that do not express the T3SS and yet cause disease. Some of these isolates employ two-partner cooperation between the type IV pili, which attach to the host cells, and a two-component toxin system, comprised of exolysin A (ExlA) and exolysin B (ExlB) (Figure 19-8). ExlB has a domain that forms a β-barrel channel in the outer membrane of the bacteria and mediates the transport of the pore-forming toxin ExlA across the bacterial outer membrane so that secreted ExlA can disrupt host cell membranes.

Resistance to Antibiotics. *P. aeruginosa* is one of the notorious ESKAPE pathogens that we talked about in chapter 16, which are so dubbed because they can effectively

Table 19-2. **Notable virulence factors of *Pseudomonas aeruginosa***

VIRULENCE FACTOR	FUNCTIONAL ROLE IN PATHOGENESIS
Flagellum (polar)	Motility, dissemination, initiation of the innate immune response
Type IV pili (polar)	Adherence, twitching motility, formation of biofilms
Lectins—LecA and LecB	Present in outer membrane, adherence to host cells, formation of biofilms
Biofilm Matrix	Protection of bacteria from hostile environments
Psl	Exopolysaccharide, attachment, structural scaffold in mature biofilms, formation of eDNA-Psl fibers that give structural support, antibiotic resistance, resistance to immunity
Pellicle (Pel)	Glucose-rich, positively charged exopolysaccharide-like cellulose, adhesin, primary structural scaffold for biofilm formation, antibiotic resistance
Extracellular DNA (eDNA)	Antibiotic resistance, extracellular DNA that forms part of matrix of biofilm
Alginate	O-acetylated exopolysaccharide polymer of (1-4)-linked β-D-mannuronate and α-L-guluronate residues; important for adherence, protection from dehydration, anti-phagocytic, contributes to biofilm structure and stability, confers resistance to antibiotics, protection of biofilm from host immune system, important for chronic infections
Bis-(3,5)-cyclic guanosine monophosphate (c-di-GMP)	Key signaling molecule that positively regulates biofilm formation and negatively regulates motility, promoting a sessile, biofilm lifecycle
Lipopolysaccharide (LPS)	
Lipid A	Endotoxic shock, sepsis
O-antigen	Serum resistance, confers serogroup specificity
Siderophores	Iron acquisition in iron-limiting environments, act as signaling molecules
Pyoverdine	Higher affinity for iron
Pyochelin	Lower affinity for iron, important for chronic infections, associated with pyocyanin
Pyocyanin	Small-molecule toxic blue-green (blue color at pH 7) pigment secreted by T2SS; redox-active zwitterionic compound that generates reactive oxygen species and has cytotoxic activity; disrupts action of cilia and phagocytic functions, causes pain and inflammation
Phospholipases	Hydrolysis of phospholipids and surfactant lipids in host membranes, cell lysis, tissue damage and invasion, phosphate acquisition, aid in iron acquisition, stimulate inflammatory responses
PLA	
PLC	
PLD	
PLH	
Hemolysins	Pore-forming toxins that damage host cell membranes
Proteases	Secreted by T2SS, involved in immune evasion, nutrient acquisition, tissue damage
LasA (Staphylolysin)	Zinc-dependent metalloprotease, proteolytic degradation of elastin and other immune proteins, staphylolytic activity
LasB (Elastase)	Proteolytic degradation of elastin, proteolytic degradation of collagen and noncollagen host proteins, degradation of opsonizing lung surfactant proteins A and D, inhibits monocyte chemotaxis
Alkaline protease	Proteolytic degradation of collagen, complement proteins C1q, C2, and C3, fibronectin, and cytokines IFNγ and TNFα
Type IV protease	Proteolytic degradation of immunoglobulins, complement proteins, surfactant protein A and protein D, plasminogen, and antimicrobial peptides
Protease IV	Proteolytic degradation of transferrin, lactoferrin, decorin, casein, and elastin
ExoA	AB toxin secreted by T2SS that ADP-ribosylates EF-2; inhibits host cell protein synthesis; causes cell death, stimulates inflammation, upregulated by iron starvation and ToxR
ExoS	Bifunctional T3SS effector protein with GTPase-activating activity that inactivates small Rho GTPases (Rho, Rac, and Cdc43) to disrupt the actin cytoskeleton and ADP-ribosyltransferase activity that modifies several host signaling proteins, including small Ras GTPases, causes cell rounding, aids in persistence and immune evasion (not found in strains with ExoU)
ExoT	Bifunctional T3SS effector protein with GTPase-activating activity that inactivates small Rho GTPases (Rho, Rac, and Cdc43) to disrupt the actin cytoskeleton and ADP-ribosyltransferase activity that modifies Crk proteins involved in focal adhesion and inhibits phagocytosis, causes cell rounding, aids in immune evasion
ExoU	T3SS effector protein that is a phospholipase A2; disrupts host cell membranes; cytotoxicity; induces inflammatory response in host (not found in strains with ExoS)

(*continued*)

Table 19-2. Notable virulence factors of *Pseudomonas aeruginosa* (*continued*)

VIRULENCE FACTOR	FUNCTIONAL ROLE IN PATHOGENESIS
ExoY	T3SS effector protein that has adenylyl cyclase activity; increases intracellular cAMP levels; causes cytotoxicity; role in pathogenesis unclear, but possibly immunomodulatory
MDR efflux system MexEF, OprMN, MexT	Antibiotic efflux pump (MexEF-OprMN) and its regulator (MexT); confers antibiotic resistance
Quorum Sensing (QS)	Three interconnected QS systems play a key role in controlling virulence factor production, biofilm formation, swarming motility, expression of antibiotic efflux pumps, and induction of a metabolic dormant state
LasR (3-oxo-C_{12}-HSL)	Acyl homoserine lactone autoinducer, LasI—acyl-HSL synthase, LasR—transcriptional activator, induces IL-8 production and inflammation in lung fibroblasts and bronchial epithelial cells
RhlR (C_4-HSL)	Acyl homoserine lactone autoinducer, RhlI—acyl-HSL synthase, RhlR—transcriptional activator, induces rhamnolipid important for biofilm formation
PQS (*Pseudomonas* quinolone signal)	Quinolone autoinducer, regulates generation of eDNA matrix component, links the Las and Rhl systems

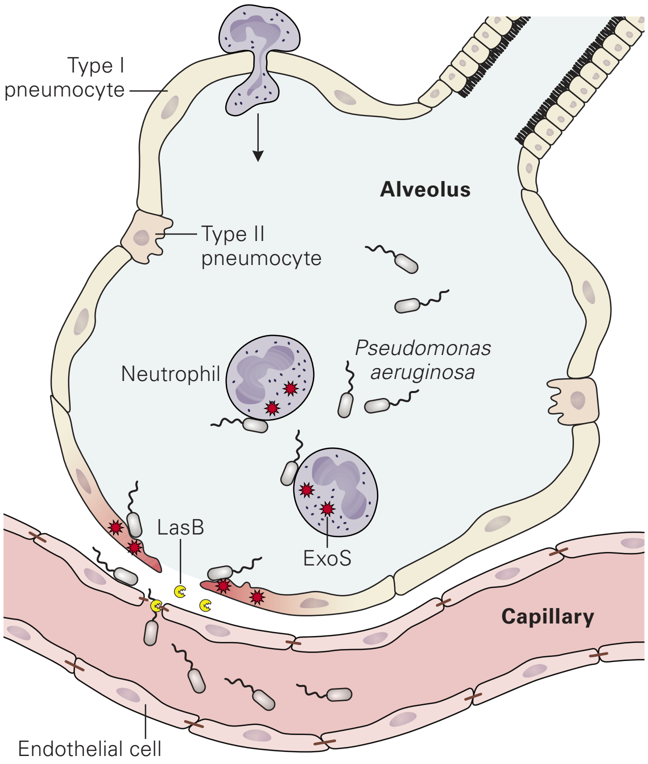

Figure 19-7. Model of *Pseudomonas aeruginosa* dissemination from the lung into the bloodstream. Following entry of certain pathogenic strains of *Pseudomonas aeruginosa* into the lower airways during lung infection, alveolar neutrophils are recruited to attempt clearance of the bacteria. However, upon contact with the neutrophils, *P. aeruginosa* instead injects the T3SS effector protein ExoS into the host cells and blocks phagocytic action. This allows the bacteria to bind to the epithelial cells and again inject ExoS into them to cause cytoskeletal changes that disrupt the epithelial barrier. The bacteria then secrete the LasB protease through the T2SS. LasB cleaves vascular E-cadherin, which disrupts the adherens junction and allows the bacteria to enter the bloodstream and disseminate through the body, causing sepsis. Adapted from Berube BJ, Rangel SM, Hauser AR. 2016. *Curr Genet* **62**:109–113, with permission.

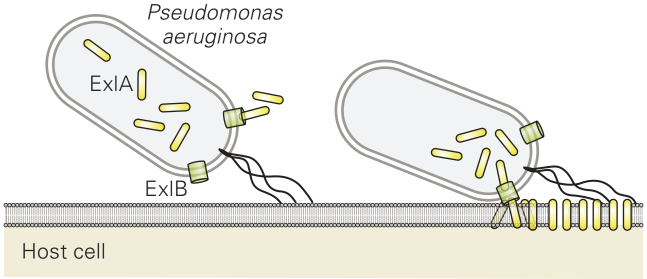

Figure 19-8. Model of cooperation between type IV pili and Exolysin A in cytotoxicity by T3SS-negative strains of *Pseudomonas aeruginosa*. The type IV pili of certain virulent isolates of T3SS-negative *Pseudomonas aeruginosa* bind to host cells to bring the secreted pore-forming exolysin A (ExlA) close to the host cell surface, resulting in host cell membrane pore formation and cytotoxicity. ExlA is secreted out of the bacteria by its cognate outer membrane transporter exolysin B (ExlB). Adapted from Basso P, Ragno M, Elsen S, Reboud E, Golovkine G, Bouillot S, Huber P, Lory S, Faudry E, Attrée I. 2016. *mBio* **8**:e02250-16, with permission.

"escape" many of the currently used antibiotics. *P. aeruginosa* is not only inherently resistant to a number of antibiotics, but many clinical strains readily acquire through HGT multiple antibiotic resistance genes during the course of infection. Thus, importantly, the bacteria have a tendency to become more resistant as the infection progresses and a biofilm is formed.

P. aeruginosa often employ a variety of different resistance mechanisms that act in concert, including expressing altered porins that prevent entry, multiple efflux systems that pump the antibiotic out of the cell, and antibiotic-inactivating enzymes. In *P. aeruginosa*, two efflux pump systems contribute to intrinsic antibiotic resistance: MexAB-OprM transports β-lactam antibiotics and MexXY-OprM transports aminoglycoside antibiotics. The genes for these pumps are under the control of the transcriptional repressor MexR. Clinical strains of *P. aeruginosa* can acquire mutations in MexR, which lead to derepression of the efflux pump genes and emergence of drug resistance. The MexEF-OprN and MexCD-OprJ efflux systems contribute to acquired resistance and can pump out a variety of drugs such as fluoroquinolones and chloramphenicol. The global transcriptional regulator MexT controls two independent signaling pathways. In one pathway, MexT represses expression of MexEF-OprN, while allowing expression of the β-lactam-permeable porin OprD that can make the bacteria susceptible to carbapenems. In the other pathway, MexT counters the quorum-sensing-mediated upregulation of MexAB-OprM and production of virulence factors, such as the genes that encode the T3SS. MexT is transcriptionally repressed by another global regulator MexS, which in environmental strains also downregulates expression of the MexEF-OprN and production of virulence factors such as rhamnolipid and pyocyanin. In clinical isolates, mutations in *mexT* or *mexS* result in overexpression of MexEF-OprN and repression of OprD.

Because the minimum inhibitory concentration (MIC) needed to kill the bacteria within the biofilm matrix is about 1,000-fold greater than that for planktonic bacteria, current efforts for treating infections are directed toward finding ways to disperse biofilms so that antibiotics can work more effectively. Unfortunately, to date there is no evidence regarding which antibiotic regimen is best to eradicate *P. aeruginosa* infection, since eradication is not usually sustained in CF patients. A new and exciting approach toward solving the CF problem that is under development is the application of targeted gene therapy for delivery of gene-editing technologies to lungs for correction of the CFTR mutation.

Burkholderia cepacia Complex—*P. aeruginosa*'s Evil Twin

BCC is a large group of related *Burkholderia* species, including *B. cepacia*, *B. cenocepacia*, and about 20 other species, typically found in aquatic or moist soil environments. Many of the traits of BCC members are similar to those of *P. aeruginosa*, including pili for adherence to various surfaces, siderophore production for iron acquisition, LPS variation, biofilm formation, and production of proteases (e.g., elastase, gelatinase) and factors that allow tissue dissemination and survival from phagocytic attack in the host. In fact, *B. cepacia* was originally called *Pseudomonas cepacia*. Moreover, BCC members resemble *P. aeruginosa* in the variety of carbon sources they can utilize. Indeed, species of both *Pseudomonas* and *Burkholderia* have been used as agents in bioremediation.

Burkholderia cenocepacia *pneumonia.* *B. cenocepacia* has been and continues to be a serious infectious disease problem in CF patients and in patients with nosocomial respiratory infections. Some patients in hospitals must have their breathing assisted by respirators, where a tube is inserted deep into the airway to deliver air to the patient. Since the tube bypasses the defenses of the upper airway, such as airflow, mucus, and ciliated cells, patients on respirators are particularly prone to develop ventilator-associated pneumonia. Also, since these patients are often comatose or semi-comatose, they are prone to the build-up of bacterial biofilms on their teeth that can increase the risk of bacteria being inhaled into the lungs, where they can cause infections. To prevent such infections, hospital staff members use mouthwash to clean the mouths and teeth of patients on ventilators. Unfortunately, batches of alcohol-free mouthwash can be contaminated with *B. cenocepacia,* so hospital staff attempting to prevent infection can actually increase the risk of infection by *B. cenocepacia.* The same thing is true for other types of hospital equipment, catheters, tubing, or multiple-dose solution vials, where aseptic technique has not been rigorously enforced.

An impressive variety of animal models, ranging from a rat model to insect and zebrafish models, are available for identifying virulence factors of BCC. With new genome technologies, it is also now possible to track parallel adaptive evolution of the bacterial species in the lungs of CF and other infected patients (Figure 19-9). As in the case of *P. aeruginosa*, the resistance of BCC to a variety of antibiotics is a serious and growing problem, which appears to be due in large part to efflux pumps that eject many types of antibiotics from *Burkholderia* cells. Antibiotic resistance is compounded in BCC by the accumulation of adaptive mutations caused by strong positive selection during infection. CF patients undergo many lengthy antibiotic treatment regimens, often without completely clearing the infection, so CF patients are at a particularly high risk of developing infections with MDR strains of BCC and *P. aeruginosa*.

Acinetobacter baumannii—A Deadly Threat Emerges from the Iraq War

A. baumannii has long been known as a common soil bacterium, but it was not previously thought to be a major cause of significant human disease. This view of *A. baumannii* dramatically changed around 2003, when during the Iraq war an alarming number of combat-injured soldiers with supposedly nonlethal flesh wounds began succumbing to life-threatening *A. baumannii* infections. The wounded soldiers brought along the Iraq strains of *A. baumannii* when they were transferred to military hospitals in Germany and then to the Walter Reed military hospital in the United States. *A. baumannii* rapidly became one of the most feared nosocomial disease-causing opportunists, spreading first to other military hospitals and then to civilian hospitals around the country. These and similar incidents with United Nations soldiers from other countries stationed in Iraq and Afghanistan helped to spread this

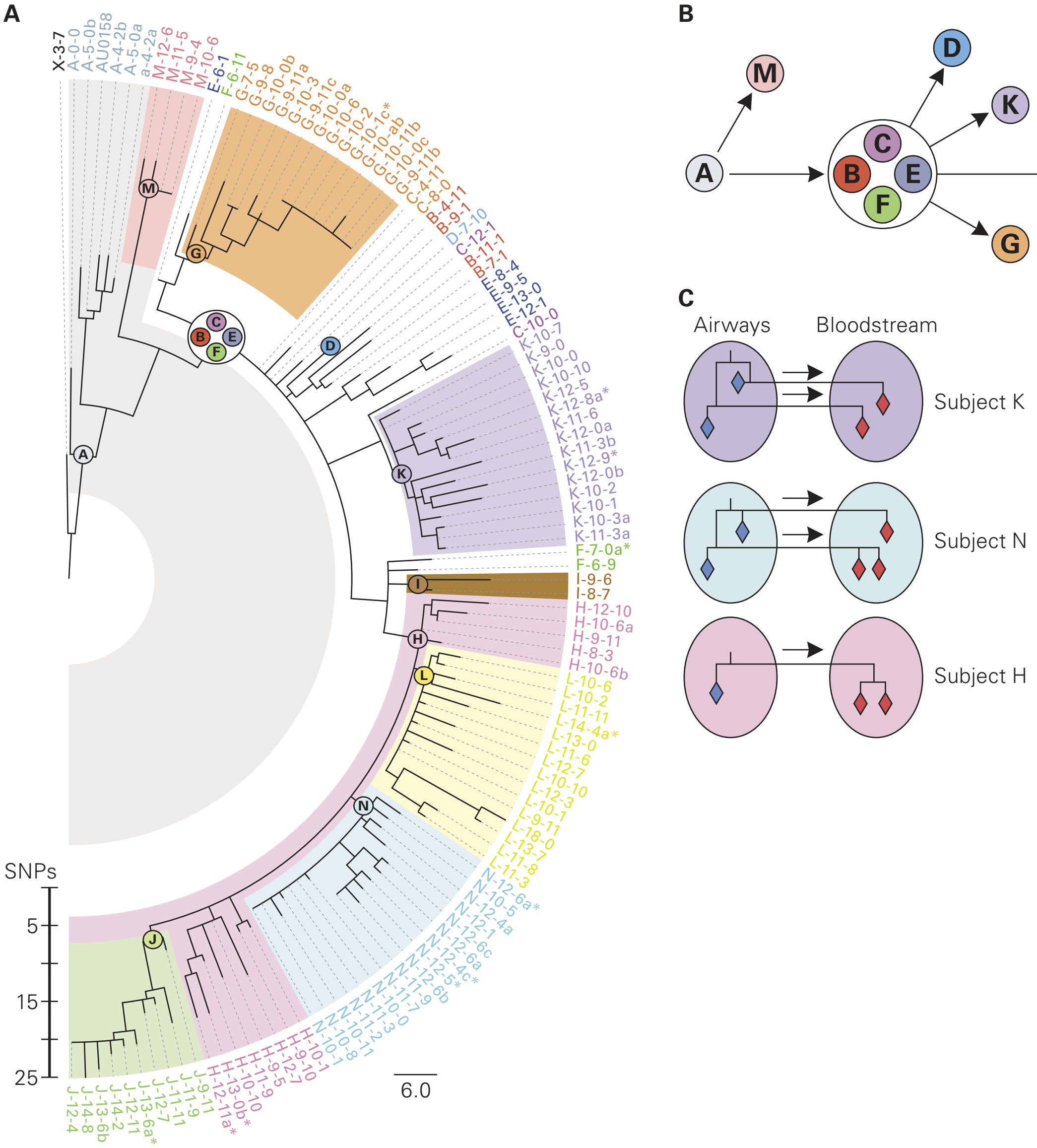
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Figure 19-9. Longitudinal traces of the the transmission of *Burkholderia dolosa* among and within cystic fibrosis patients. **(A)** Phylogenetic tree showing the relationship among *B. dolosa* isolates from 112 CF patients, labeled according to patient (letter) and time of sampling (years and months after first isolate). Shading is color-coded according to patient. Small letters indicate multiple samples taken from the same patient at the same time. The last common ancestor of the isolates is indicated by a circle with the appropriate color and label. Blood isolates are indicated by asterisks. **(B)** Transmission network based on phylogenetic relationships inferred from last common ancestor. **(C)** Transmission network between blood and lung isolates from the same patient, for patients suffering also from bacteremia (subjects H, K, and N). Redrawn from Lieberman TD, Michel JB, Aingaran M, Potter-Bynoe G, Roux D, Davis MR Jr, Skurnik D, Leiby N, LiPuma JJ, Goldberg JB, McAdam AJ, Priebe GP, Kishony R. 2011. *Nat Genet* **43:**1275–1280, with permission.

new scourge around the world. In point of fact, *A. baumannii* has decidedly earned its mark as an ESKAPE pathogen (see chapter 16).

At the time of it first emergence as a threat, very little was known about the virulence factors of these new strains of *A. baumannii,* but now it is clear that they are intrinsically virulent when a new group of susceptible hosts presents itself, such as wounded soldiers or immunocompromised hospital patients. Recent comparative genomic analyses have identified a number of virulence factors, including several porins involved in adherence, invasion, and persistence; pili and filamentous hemagglutinin for adherence and biofilm formation; capsular polysaccharide for biofilm formation and in vivo survival; modified LPS conferring serum resistance; phospholipases (PLC and PLD) for serum resistance, invasion, and in vivo survival; several metal ion acquisition systems for iron, zinc, and manganese; T2SS for in vivo survival; T5SS for adherence and biofilm formation; T6SS for host colonization and bacterial competition; and other secreted virulence factors for killing host cells.

Antibiotic Resistance. Concern about *A. baumannii* extends beyond its ability to cause serious wound infections. What makes these new *A. baumannii* strains so dangerous is the fact that they are also remarkably difficult to treat due to extensive inherent and acquired antibiotic resistance. Indeed, *A. baumannii* proved to be one of the multidrug resistant pathogens that are resistant to so many antibiotics that they have been called "**panresistant**" and are now categorized as one of the "**superbugs**," along with MRSA and CA-MRSA (chapter 18) and *Mycobacterium tuberculosis*, so dubbed because they are not only MDR but **XDR (extensively-drug resistant)**. There are still a few first-line and some second-line antibiotics that work, but their number is small and dwindling (e.g., carbapenem, tigecycline, and colistin resistance is rising), making treatment of *A. baumannii* infections or clearance of carriers no simple undertaking. Added to this antibiotic resistance is the inherent resistance of the bacterium to disinfectants and various environmental conditions, which hamper containment and decontamination and necessitate stringent infection-control procedures such as aggressive sterilization and patient isolation (implementation of the so-called 1 patient = 1 nurse/doctor rule) in hospitals during incidents. Some hospitals have even been forced to close entire units in order to interrupt transmission. In total, the costs associated with MDR/XDR *A. baumannii* infections are exceptionally high as a result of the additional transmission control measures, decontamination procedures, and administration of multiple second-line intravenous antibiotics that must be done to reduce spread. Research efforts are now turning toward alternative strategies such as bacteriophage or bacteriolysin-based therapies (see chapter 16).

Don't Forget the Arthropods!

Ehrlichia spp.

Arthropod-borne infections are well known. Major bacterial arthropod-borne diseases, such as plague (caused by *Yersinia pestis* via fleas), Rocky Mountain spotted fever (caused by *Rickettsia rickettsii* via ticks), and Lyme disease (caused by *Borrelia burgdorferi* via ticks), have received a lot of attention over the years. All of these arthropod-borne pathogens breach the host innate defenses and get into the human body via an arthropod bite (e.g., from a tick, flea, louse, or mite). Even though most of these pathogens are not usually considered opportunists because they cause infections in otherwise healthy people, some of them could be considered opportunists in the sense that they only cause serious disease in immunocompromised individuals. An example of such an arthropod-borne opportunistic pathogen is *E. chaffeensis*, which is transmitted by ticks, principally the lone star tick (*Amblyomma americanum*). The geographical distribution of ehrlichiosis cases, which occur primarily in the southeast of the United States, is probably due to limitations in the range of the lone star tick. *E. chaffeensis* is an obligate intracellular pathogen, most closely related to the *Rickettsia* spp. (the causative agents of Rocky Mountain spotted fever and typhus). It invades monocytes and causes a febrile disease called **human monocytotrophic ehrlichiosis (HME)**.

The reason for calling *E. chaffeensis* an opportunist is that HME is seen almost exclusively in older people and in people with compromised immune systems. In fact, the early cases of HME occurred primarily in golf-centered communities in Florida and Georgia, where most of the residents were elderly people who spent time on the golf course, where they could easily come into contact with ticks—especially if they spent a lot of time in the rough chasing after wayward golf balls. Since then, however, HME has been seen in a wider range of people, but *E. chaffeensis* still seems to have a predilection for individuals with waning ability to fight off bacterial invaders.

E. chaffeensis is maintained in the environment in nonhuman mammals, mainly the white-tailed deer (*Odocoileus virginianus*), but other animals can serve as reservoirs (goats, raccoons, dogs, and coyotes). In this respect, its life cycle resembles that of *Borrelia burgdorferi,* the cause of Lyme disease. In both cases, humans seem to be an accidental host, not a host that provides an important reservoir for the bacteria. In humans, *E. chaffeensis* invades monocytes or macrophages, somehow prevents phagosome-lysosome fusion, and multiplies within the phagosome. Ultimately, the bacteria are released and go on to infect other mammalian cells. *E. chaffeensis* is a tiny bacterium with a Gram-negative type cell wall that is not easily visible in Gram-stained preparations. New approaches to label the peptidoglycan cell wall by covalently incorporating fluorescently labeled D-amino acids in situ have shown promise in detecting some small, hard-to-detect extracellular and intracellular pathogens.

The discovery of *E. chaffeensis* as an arthropod-borne opportunist raises the question of whether there are other such arthropod-borne bacterial opportunists. Possibly, the pathogenic potential of the arthropod-borne bacterial pathogens ranges over a continuum as wide as that seen in free-living bacteria. It will be interesting to see if other cases of arthropod-borne opportunists—if you accept the characterization of *E. chaffeensis* as an opportunist—will be discovered. Another area of future research is how these pathogens interact with each other during co-infections of the arthropods or their mammalian prey.

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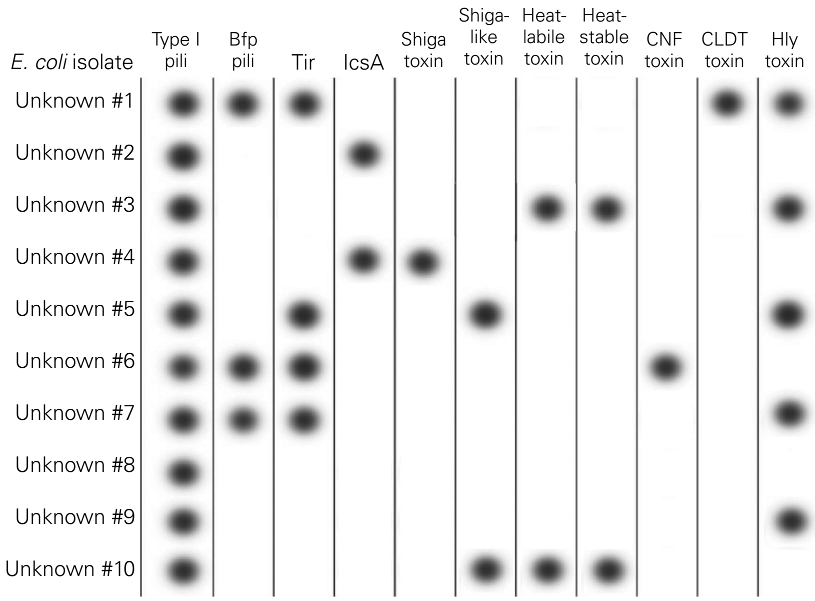
Questions

1. Speculate about how and why different opportunists seem to be specifically adapted to take advantage of a limited range of opportunities.
2. How would you formulate Koch's postulates for opportunistic pathogens?
3. In the case of the *E. coli* K strains, why would the number of acyl chains on *E. coli* LPS affect the function of a TLR, given that LPS does not bind the TLR directly?
4. Why is there no vaccine against *E. coli* neonatal meningitis?
5. Would it be appropriate to develop a vaccine against *B. fragilis* or *P. gingivalis?* Why or why not? If not, what would be the most effective types of prevention?
6. Speculate about why *P. aeruginosa* and *B. cenocepacia* seem to outdo organisms found in the human body when it comes to infecting the lungs of a cystic fibrosis patient.
7. Why might opportunists, such as *P. aeruginosa, B. cenocepacia,* and *A. baumannii,* produce so many different virulence factors? Why is cell-to-cell signaling that controls production of many of these factors so important? Could these signaling systems become a target for antibiotics?
8. Do you consider *E. chaffeensis* an "opportunist"? Give arguments for and against this designation. If you accept *E. chaffeensis* as an opportunist, what implications does this have for other possible arthropod-borne microbes?
9. EHEC and EPEC strains are often confused with each other and misidentified. For each of the following, describe at least two of the main similarities or differences between these strains in regards to:
   a. interactions with host cells.
   b. production of toxins.
   c. symptoms of infection.

Solving Problems in Bacterial Pathogenesis

1. You are a scientist working for the FDA, and you have been given the task of designing a virotyping kit that would allow you to readily identify pathogenic strains of EHEC, EIEC, EPEC, ETEC, and *Shigella* based on probes that recognize virulence factors. Probes to which specific virulence factors would you choose to use in your strain-virotyping kit? Be sure to pick *sufficient* and *unique* factors that will allow each of the strains to be *clearly* and *definitively* identified from the others. Give your rationale!
2. Working for the EPA, you have isolated several *E. coli* strains contaminating a pond near a sewage treatment facility. Using an antibody-based diagnostic kit, you have obtained the results below on your dot-blot (similar to a Western blot, only the protein sample is directly applied as a spot to the membrane without first running SDS-PAGE). Based on the results shown in Figure 1, predict the identity of each of the unknown isolates. Be sure to *briefly* state your rationale.



E. coli isolate	Type I pili	Bfp pili	Tir	IcsA	Shiga toxin	Shiga-like toxin	Heat-labile toxin	Heat-stable toxin	CNF toxin	CLDT toxin	Hly toxin
Unknown #1	●	●	●							●	●
Unknown #2	●			●							
Unknown #3	●						●	●			●
Unknown #4	●			●	●						
Unknown #5	●		●			●					●
Unknown #6	●	●	●						●		
Unknown #7	●	●	●								●
Unknown #8	●										
Unknown #9	●										●
Unknown #10	●					●	●	●			

Figure 1. Dot blot.

Special Global Perspective Problems: Integrating Concepts in Pathogenesis

Problem 1

You are a microbiology graduate student with aspirations of one day completing your doctoral work in bacterial pathogenesis. Your research thesis advisor has ample funds and has allowed you to pursue your own project from scratch. While contemplating what bacterium you would like to work on, a group of your friends return from spending holiday break at a resort in southern Florida. Shortly after returning home, five of these friends, who had enjoyed the spa's Jacuzzi facilities, presented to the student health center with an acute generalized eruption of follicular lesions (skin rash with pustules over most of the body). The doctor prescribed some antibiotic ointment containing a combination of gentamicin and Augmentin (a combination of the β-lactams amoxicillin and clavulanic acid). After one week of topical treatment, three of the five friends healed without sequelae. However, two of them had sustained some abrasions at a volleyball game played during the vacation. Despite treatment with the antibiotic ointment, their wounds became further infected with symptoms of pain, swelling, and redness followed a couple of days later by fever and then admission into the hospital with symptoms of septicemia and disseminated intravascular coagulation (DIC). Intravenous administration of gentamicin along with some surgical debridement of the wound area rescued these two friends from a potentially dire outcome. Bacterial cultures from the blood of these two friends revealed that the septicemia was caused by a Gram-negative, rod-shaped bacterium, which looked similar to the organism observed in Gram stains from the follicular lesions. Having piqued your desire to prevent this from happening again (and fulfill a vendetta on behalf of your friends against this pathogen), you decide to examine this bacterium in more depth, and so you obtain a culture of the blood isolate from the diagnostics laboratory. You isolate the genomic DNA and sequence the 16S rRNA gene to determine that the bacterium is distantly related to *Pseudomonas* and *Aeromonas* species, which are largely found in aquatic environments but can cause opportunistic infections. Below are a series of experiments you then undertake to study the pathogenesis of this new bacterium, which you have named *Graduamonas exhaustmium*.

1. A combination of gentamicin and Augmentin was used for the follicular lesions, but only gentamicin was used for the septicemia.
   a. Why was Augmentin not used intravenously in this case, yet gentamicin was?
   b. Why is Augmentin formulated as a combination drug?
   c. Why was surgical debridement necessary?
   d. What caused the symptoms of DIC in this case? Be specific.

2. Although you do not have a culture of the bacterium from the follicular lesions, you suspect that the bacterium observed in the Gram stains is the same *G. exhaustmium* isolated from the blood samples.
   a. What four criteria must be satisfied to show that *G. exhaustmium* is indeed responsible for causing the follicular lesions?
   b. Describe how you would go about demonstrating this experimentally. Provide a reasonable model of infection. Be specific for this case.
   c. Provide one additional, different molecular experimental approach that could be performed to help satisfy these criteria for this case.

3. Using your experimental model of infection, you wish to identify potential virulence factors. Briefly describe how you might go about doing this. Provide your rationale for choice of approach. Be sure to include how you would verify that they are indeed virulence factors.

4. From your screening, you isolate 10 mutants with deletions in genes that encode putative virulence factors (denoted as GeMut1-GeMut10). Sequence analysis of the genes reveals that Mut1 has an N-terminal stretch of 15 hydrophobic residues but otherwise no homology motifs to any known proteins. Mut2 has four membrane-spanning helices plus some homology to flagellar motor protein. Mut3 has homology to Rho GTPase-activating proteins. The gene for Mut4 is in the same operon as *mut3* and is a short gene with some DNA sequence complementary to the 5' region of *mut3*. Mut5 has several membrane-spanning hydrophobic helices and some homology to the protein SecY. Mut6 has an N-terminal stretch of 15 hydrophobic residues, followed by several membrane-spanning hydrophobic helices and a C-terminal domain with homology to cysteine proteases. The genes for Mut7-Mut10 are all part of the same operon. Mut7 has several membrane-spanning hydrophobic motifs and a C-terminal domain with homology to protein kinases. Mut8 has homology to LuxI. And Mut9 has a C-terminal domain with homology to DNA-binding proteins. Mut10 has an N-terminal stretch of 12 hydrophobic residues and a C-terminal domain with homology to glycosyltransferases. To gain some insight into the role of these virulence factors in pathogenesis, you decide to conduct additional experiments. First you perform a gentamicin protection assay and plaque assay using human cultured fibroblast-like cells. The results are shown in Table 1.

a. Interpret these results with rationale.
b. Predict the function of the virulence factor encoded by the gene *mut1*.
c. Provide one additional, different experiment that could be performed to confirm your prediction.

5. In the next experiment you grow the bacteria in medium and then filter the bacterial culture and treat cultured host fibroblastic cells with either the bacteria or with the filtered bacteria-free medium. The results are shown in the Figure 2 below.
   a. Interpret with rationale these results.
   b. Predict the function of the virulence factors encoded by the genes *mut2-mut4*.
   c. Provide one additional, different experiment that could be performed to confirm your predictions for each.

6. You find that the wild-type bacterium elicits a very strong host humoral immune response, yet wild-type bacteria appear to be able to evade this response and cause septicemia, even in hosts with prior exposure to the bacterium. You find that GeMut5 and GeMut6, while able to colonize and form follicular lesions, are no longer able to cause septicemia and are quickly cleared from the blood stream in hosts with prior exposure to the bacterium.
   a. Describe how *G. exhaustmium* antigens such as Mut1 are presented to the host immune system to result in a strong humoral response.
   b. Predict with rationale a possible function for Mut5 and Mut6.
   c. Provide one additional, different experiment that could be performed to confirm your prediction.

7. You find that the wildtype bacterium and GeMut2-GeMut6 form mucoid colonies on agar plates and stain purple with crystal violet dye when grown in test tubes (as shown in Figure 3). The mutants GeMut1 and GeMut7-GeMut10 no longer have this property. But, if you add bacterial culture filtrates from GeMut7 and GeMut9 to cultures of GeMut8, but not GeMut1 or GeMut10, GeMut8 will stain with crystal violet.

Table 1.

	GENTAMICIN ASSAY (NO. OF COLONIES)		PLAQUE ASSAY (NO. OF PLAQUES OBSERVED)
BACTERIUM	WITHOUT GENTAMICIN	WITH GENTAMICIN	
Wild type and GeMut2 to GeMut10	10^9	10	None
GeMut1	10	10	None

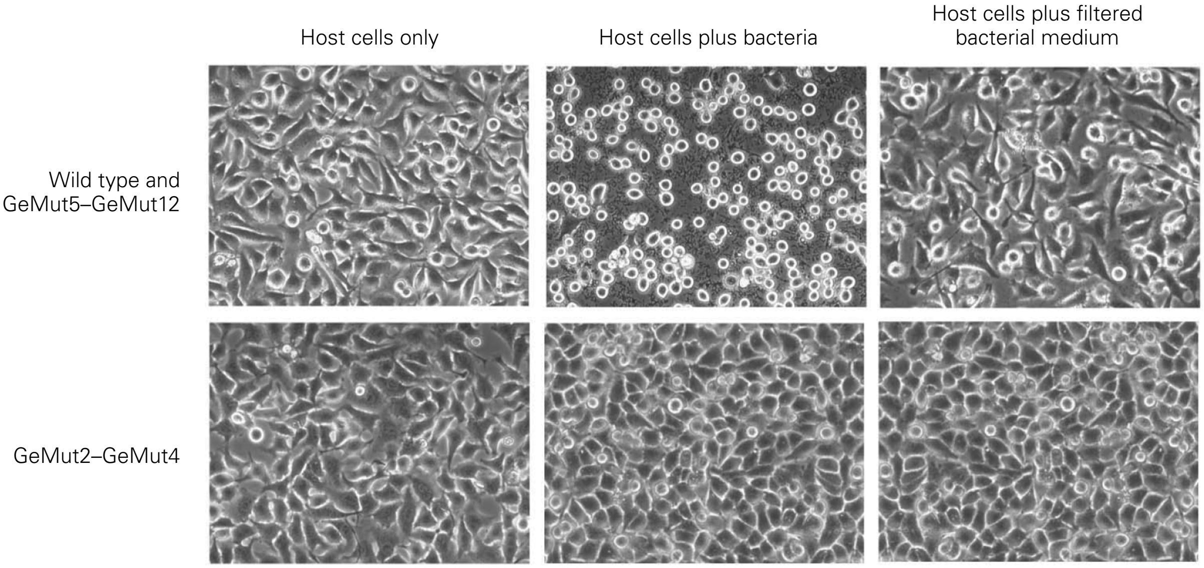

Host cells only
Host cells plus bacteria
Host cells plus filtered bacterial medium
Wild type and GeMut5–GeMut12
GeMut2–GeMut4

Figure 2. **Results of host fibroblast experiment.**

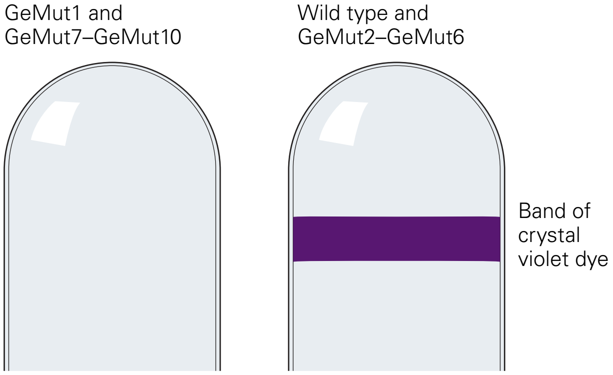


Figure 3. **Crystal violet biofilm assay.**

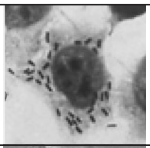
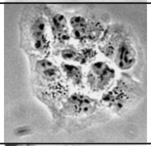
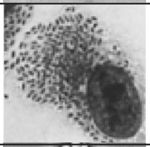
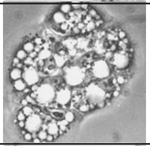
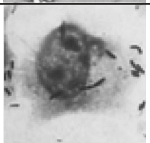
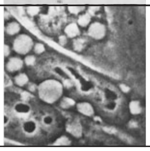
a. Interpret with rationale these results.
b. Predict the function of the virulence factors encoded by the genes *mut7–mut10*.
c. Provide one additional, different experiment that could be performed to confirm your predictions for each.

Problem 2

There have been a number of recent high-profile incidences that illustrate the havoc, alarm, and economic consequences that can result from widespread distribution of contaminated food due to accidental introduction of harmful microbes during food processing. What previously was seen only sporadically, such as at family gatherings or community picnics, moved abruptly into the public's eye with the large-scale problem of food-borne pathogens. These dangerous microbes have been associated with hundreds of multistate or multination outbreaks of contaminated food. Late in the summer, contamination of prepackaged fresh spinach with *E. coli* O157:H7, a toxin-producing bacterium that causes dysentery-like diarrhea and can cause kidney failure and death, especially in children and the elderly, led to 204 cases of illness across 26 states, with 104 hospitalizations, 31 kidney failures, and 3 deaths. The outbreak, which was traced back to spinach obtained from a few fields in California, shook consumer confidence and cost the industry an estimated $150M in economic losses. This has also prompted fear that a deliberate act of bioterrorism could result in even worse consequences. In response to the growing need for better food surveillance, the FDA and USDA have implemented several joint surveillance and risk assessment programs for prevention and detection of food-borne diseases. As part of these food-safety programs, a group of researchers at one of the testing laboratories has isolated six different strains of bacteria associated with two recent outbreaks of severe diarrheal disease, the source of which was traced back to two large food processing plants, one in the Midwest (strains #1–3) and the other in New England (strains #4–6). All of the strains had LD_{50} values of ~10 in a piglet model of intestinal diarrhea, in which bloody diarrheal stool was observed. Being interested in understanding the pathogenesis of these new strains, the researchers subjected each of these strains to a series of experiments to identify these strains and to determine what virulence factors they might have.

1. Based on the results shown in Table 2, the researchers ask you, as an expert consultant in bacterial pathogenesis, to predict the possible virulence factor(s) that must be present (or absent) in strains #1–6. Be sure to state your rationale. (Note: Be complete, but base your answer only on the information provided from the assays used in the table.)
2. Because of the urgency involved in characterizing these strains, the researchers plan to perform Tn-Seq and genome sequencing to identify the genes encoding the virulence factors that you predict are associated with each of these strains. They also ask you to provide at least one additional biological or cell-based experiment (not involving sequencing) for each strain that they could perform to confirm the predictions. Be sure to state how the data will be visualized or measured, what reagent(s) and instrument(s) are needed, and what the expected results will be for each experiment. (Note: For strains predicted to have multiple virulence factors, you may need more than one type of experiment to verify all of your predictions.)
3. In your expert opinion as a microbiologist and researcher in the field of bacterial pathogenesis, is there plausible cause for the researchers to call the Centers for Disease Control and Prevention (CDC) and the Department of Homeland Security (DHS) regarding the possibility that either of these incidences described in Problems 1 or 2 could be an act of bioterrorism? Be sure to state your rationale.

Table 2.

	16S rRNA Phylotype	**Gentamicin Assay**			**Toxic Shock Assay**	**Plaque Assay**	**Host cell Morphology Assay**	**Acid Tolerance Assay**
Strain	16S rRNA gene BLASTed against DNA database	without gentamicin (cfu/mL)	with gentamicin (cfu/mL)	with gentamicin and mannose (cfu/mL)	High levels of inflammatory cytokines (TNF & IL-1) in blood	Phenotype observed after 24 hr	Microscopic image of gram-stained intestinal epithelial cells incubated 8 hr with bacteria	# of live bacteria after 3 hr at pH 2 (cfu/mL)
#1	*Listeria monocytogenes*	10^9	10^8	10^8	no	Large plaques		10
#2	*E. coli*	10^9	10^2	10^2	yes	All clear		1
#3	*E. coli*	10^9	10^8	10^2	no	Small plaques		10
#4	*E. coli*	10^9	10^2	10^2	yes	All clear		10^{12}
#5	*Bacillus* species	10^9	10^8	10^8	yes	Large plaques		10^{12}
#6	*Helicobacter pylori*	10^9	10^2	10^2	yes	No plaques		10^{10}

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CHAPTER 20

The Changing Roles of Microbiologists in an Age of Bioterrorism and Emerging Diseases

Any military strategist will tell you that the best defense against a threat is information; hence, intelligence gathering is one of the most critical components of any combat operation. Similarly, the best defense against an infectious disease threat is information—information that is readily accessible and understandable by all parties and that enables those parties to assess the risk, formulate response options, and then react calmly and rationally to the threat.

When Microbiologists Are Called to the Front Line

The concept of biological warfare or bioterrorism, i.e., the deliberate use of biological agents (microorganisms or their toxic products) to further nefarious goals, is not new. Historically, there are numerous accounts of the use of toxic, diseased, or contaminated items being used to sicken, weaken, or kill an enemy. In ancient times, armies were known to poison enemy water wells with rye ergot or purgative herbs (hellebore, skunk cabbage) or diseased animal and human corpses. Archers would dip their arrows in poison, manure, or diseased blood. In medieval times, armies would use catapults to send disease-causing cargo over castle walls, including manure, dead horses that died from diseases such as glanders or anthrax, or even bodies of humans who had died from plague or smallpox. Enemies would lace wine with the blood of leprosy patients, foxglove, hemlock, or other poisons. In more recent history, blankets and linens contaminated with smallpox were given to Native Americans during wartime, such as those purportedly given the Delaware and Shawnee Indians by British troops during the siege of Fort Pitt in the summer of 1763. British forces during the American Revolutionary War gave smallpox to besieged civilians in Boston, and in 1776, Benedict Arnold was accused, among other things, of deliberately spreading smallpox throughout the Continental Army.

In modern times, biological warfare, along with chemical warfare, escalated to new and terrifying heights, becoming ever-more sophisticated and grander with each world war. During World War I, biological weapons were mostly directed against animals. Anthrax spores from *Bacillus anthracis* or water contaminated with *Burkholderia mallei*, the cause of glanders, were used to infect horses and mules, which hampered troop and artillery movements and supply convoys. The horrific ramifications evidenced by the use of these chemical and biological agents during World War I led to the Geneva Protocol in 1925, which banned any further use (but alas not possession, research, or development) of such bioweapons. However, this resolution was all too quickly set aside during World War II with the establishment of extensive bioweapons programs that rapidly switched from defensive to offensive measures and sometimes involved crimes against humanity. By the end of the war, most developed countries had full-blown, active bioweapons programs that continued to expand during the Cold War years.

It was not until 1969 that the United States officially terminated its offensive bioweapons program, destroying all stockpiles and switching back to defensive measures. The first international disarmament treaty banning the development, production, and stockpiling of biological and toxin weapons was initiated in 1972 and ratified by 22 governments in 1975 at the **Biological and Toxins Weapons Convention** (abbreviated as **BWC** or **BTWC**), to which over 176 country-states are now party. Unfortunately, the lack of a formal verification regime for monitoring compliance limited the effectiveness of the BTWC; indeed, it became clear in the 1980s and 1990s that some state-sponsored bioweapons programs, in particular those of Iraq and the Soviet Union, had remained active and offensive.

The 1980s and 1990s also saw several smaller-scale incidents involving intentional release of biological agents by non-state-sponsored entities. In 1984, the Rajneeshee religious cult contaminated salad bars in Oregon with *Salmonella enterica* serovar Typhimurium in an attempt to influence the outcome of a local election. This incident resulted in hundreds of cases of gastroenteritis but no deaths. Japan's apocalyptic Aum Shinrikyo cult tried numerous times in the early 1990s to spray anthrax spores around Tokyo. But these attempts failed, presumably because the strain of *B. anthracis* used was avirulent. In 1995, the group turned instead to the release of sarin gas in a Tokyo subway, which killed 12 commuters, seriously injured 54, and affected thousands.

Despite these precedents, the use of the U.S. postal service as a vehicle to spread anthrax spores in a deliberate act of bioterrorism, particularly in the wake of the 9/11/2001 attacks on the World Trade Center in New York and the Pentagon Building in Washington, D.C., came as a major surprise to the general public and government and health officials, whose mindset about the use of bioweapons was still mired in large-scale attacks. Amazingly, of the tens of thousands of people that were potentially exposed, there were only 22 documented cases of anthrax attributed to the mailings, thanks to the rapid containment and prophylactic measures taken once the threat was realized. Moreover, only five of the 11 victims who actually developed symptomatic inhalation anthrax died. Although tragic, this death toll was far below the 100% death rate that almost certainly would have resulted without rapid medical intervention using today's medical advances.

The 2001 anthrax attacks marked a major paradigm shift in our way of thinking about how and from where potential bioterror acts might occur and what our responses currently are and will need to be to prevent another such attack in the future (Table 20-1). No longer is a bioweapon only considered an implement of a state-sponsored war conducted on a grand scale, as a so-called **"weapon of mass destruction" (WMD)**, but rather it is just as likely to be used on a smaller scale by groups or individuals to spread terror and disrupt normal routine operations, more as a **"weapon of mass disruption"** (still a WMD). As a consequence of the pivotal events of 2001, defense against bioterror agents, collectively dubbed "**select agents**" (Table 20-2), emerged as a major global health priority in the United States and elsewhere. The United States alone spent many billions of dollars toward this effort during the first 10 years after the event and today continues to spent hundreds of millions of dollars toward combating select agents and other biological agents considered to be threats (**biothreats**).

In this new era, where biothreat agents, such as anthrax spore-containing powders and botulinum neurotoxin-laced milk, could be introduced as stealth biological agents to incite terror in the general public and cause massive disruption of daily life, microbiologists have become an important and integral part of the homeland defense network. From the outset, microbiologists have always worked closely with the military, the CDC, the FBI, and other law enforcement agencies, as well as the more recently instituted Department of Homeland Security, to detect, identify, trace, monitor, decontaminate, treat, and prevent potential bioterror events and to inform and educate authorities and the public.

Tracking Down a Bioterrorist

Bioterrorism poses unique challenges for security due to the intrinsic differences between an act of releasing a biological agent and, say, building and detonating a bomb or even releasing a chemical, such as a nerve gas. The explosive ingredients used to build a bomb are obvious, readily identifiable, and often unmistakable in their intent. There is only one purpose for which a person might build a bomb, and that is to detonate it and cause destruction, and to poten-

Table 20-1. Sources of biological threats

NATURAL BIOLOGICAL THREATS	BIOLOGICAL THREATS ORIGINATED BY PEOPLE
Persistent, ever-present infectious diseases	Accidental release of infectious agents
High-impact infectious diseases (major health, ecological, and economic consequences)	Unintentional release through mishap or malfunction
New and reemerging infectious diseases	Anthropogenic impact on environment or ecology that shifts natural habitats or exposes new sources
Multidrug-resistant pathogens	Nonmalicious but intentional release or introduction that results in an unintended outcome
Human-to-human transmission	Sloppiness or negligent hygiene during food preparation or processing
Vector-borne, epizoonotic transmission	Deliberate (the "terror effect")
Foreign animal, zoonotic transmission	State-sponsored bioweapons: WMD, or "weapons of mass destruction"
Food-borne transmission	Bioterrorism acts by groups or individuals: WMD, or "weapons of mass disruption"
Water-borne transmission	
Increasing populations of people with weakened immune systems due to infectious diseases, chronic diseases, and aging	
Invasive alien species	
Plants	
Animals	
Insects	

tially harm people or things nearby. The nature and uses of a chemical substance are likewise readily identifiable, and the potential toxicity of the substance is usually well known or predictable. It is also obvious that the presence of any sizable quantity of the toxic substance, especially in an unexpected location, is likely to have but one purpose—again, to cause damage and potential harm. The same cannot always be said about a biological agent.

Unintentional or Deliberate?

The presence of a biological agent or the isolation of a biological agent from an infected individual does not necessarily mean that the biological agent was deliberately introduced to cause harm. Disease could be caused from natural exposure to a biological agent or through accidental, nonmalicious exposure. Biological agents are often used for beneficial biomedical research to help develop therapeutics or for educational purposes to learn about biological processes. Hence, biological agents are often referred to as "**dual-use agents**" with both helpful and potentially harmful applications. An example is the *Clostridium botulinum* neurotoxin (see chapter 12), which is not only a highly potent paralyzing and potentially lethal toxin, but also a cosmetic for wrinkles (sold commercially as Botox) and a therapeutic agent used in medicine to aid in wound healing and to treat nerve disorders, such as dystonia (facial tics caused by involuntary muscle spasms), incontinence, and other neuropathies. Complicated issues arise when assessing research that involves dual-use agents that could be misused by some individuals to pose a significant threat to public health and safety. Since 2015, the U.S. government has had policies in place devoted to ensuring that research practices and procedures are implemented at the institutional level to identify **dual-use research of concern (DURC)** and that measures are taken to minimize risk of their use for nefarious purposes.

In some cases, deliberate release of a biological agent is clear-cut. For example, an outbreak of smallpox, caused by the variola virus, nowadays can only be attributed to a deliberate release because human smallpox has been eradicated globally since 1980, and there are only two known stocks of smallpox still in existence in high-level biocontainment facilities at the CDC and in Koltsovo, Russia. Similarly, epidemic outbreaks of inhalation anthrax are very rarely seen in developed countries and can likely be attributed to deliberate or accidental release of a significant number of spores into the atmosphere. For example, epidemiologists and other experts around the world were convinced that the deadly 1979 anthrax incident in Sverdlovsk (today known as Yekaterinburg), Russia, was due to a massive release of anthrax spores from a Soviet military bioweapons facility. About 100 people (the exact number is unknown because records were purportedly destroyed) and numerous animals downwind of the facility were killed. Since anthrax is not contagious (i.e., it is not spread from individual

Table 20-2. List of United States Department of Health and Human Services (HHS)/CDC and USDA select agents and toxins (as of November 2017)

	MEMBERS			
GROUP	**TOXINS**	**BACTERIA**	**VIRUSES**	**FUNGI**
HHS select agents and toxins	Abrin Botulinum neurotoxins[a] Botulinum neurotoxin producing species of *Clostridium*[a] Conotoxins (Short, paralytic alpha conotoxins containing the following amino acid sequence X_1CCX_2PAC-$GX_3X_4X_5X_6CX_7$) Diacetoxyscirpenol Ricin Saxitoxin Staphylococcal enterotoxins A, B, C, D, E subtypes T-2 toxin Tetrodotoxin	*Bacillus cereus* Biovar *anthracis* *Coxiella burnetii* *Francisella tularensis*[a] *Rickettsia prowazekii* *Yersinia pestis*[a]	Crimean-Congo haemorrhagic fever virus Eastern Equine encephalitis virus Ebola virus[a] Kyasanur Forest disease virus Lassa fever virus Lujo virus Marburg virus[a] Monkeypox virus Omsk hemorrhagic fever virus Reconstructed replications competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (Reconstructed 1918 Influenza virus) SARS-associated coronavirus (SARS-CoV) South American Haemorrhagic Fever viruses: Chapare Guanarito Junin Machupo Sabia Tick-borne encephalitis complex (flavi) viruses: Far Eastern subtype Siberian subtype Variola major virus (Smallpox virus)[a] Variola minor virus (Alastrim)[a]	
Overlap select agents and toxins		*Bacillus anthracis*[a] *Bacillus anthracis* Pasteur strain *Brucella abortus* *Brucella melitensis* *Brucella suis* *Burkholderia mallei*[a] *Burkholderia pseudomallei*[a]	Hendra virus Nipah virus Rift Valley fever virus Venezuelan equine encephalitis virus	
USDA select agents and toxins		*Mycoplasma capricolum* *Mycoplasma mycoides*	African horse sickness virus African swine fever virus Avian influenza virus Classical swine fever virus Foot-and-mouth disease virus[a] Goat pox virus Lumpy skin disease virus Newcastle disease virus Peste des petits ruminants virus Rinderpest virus[a] Sheep pox virus Swine vesicular disease virus	
USDA plant protections and quarantine (PPQ) select agents and toxins		*Ralstonia solanacearum* *Rathayibacter toxicus* *Xanthomonas oryzae*		*Peronosclerospora philippinensis* (formerly *Peronosclerospora sacchari)* *Coniothyrium glycines* (formerly *Phoma glycinicola* and *Pyreochaeta glycines*) *Sclerophthora rayssiae* *Synchytrium endobioticum*

[a] Denotes Tier 1 Agent. These agents are considered to present the greatest risk of deliberate misuse with severe consequences, and thus pose a severe threat to public health and safety.

to individual) and there were many people and animals in the same area affected, the outbreak had to be caused by a massive release of spores. Although Soviet officials denied it at the time, insisting that it was a natural outbreak, it was later confirmed, after the dissolution of the Soviet Union in 1992, that the outbreak was indeed caused by the accidental (man-made) release from the Sverdlovsk facility.

On the other hand, an epidemic outbreak of plague (caused by *Yersinia pestis*) or monkey pox (similar to but not the same as human smallpox), both of which are contagious and have natural reservoirs, can be caused by natural exposure to infected animals or individuals instead of by a deliberate, malicious act. Questions, but no real scientific evidence, have arisen over the origins of the SARS (severe acute respiratory syndrome caused by coronavirus) and Ebola (hemorrhagic fever caused by Ebola virus) outbreaks, which suddenly emerged and rapidly spread among human populations. Some have even speculated about whether these bioagents were used by bioterrorists to spread fear. The problem with these scenarios is that it remains hard to conclusively discern involvement of foul play.

Timing

The devastating effects of detonating a bomb are experienced immediately. Similarly, the toxic effects of a chemical weapon are often immediately evident. This gives the perpetrators very little time to escape and helps the authorities better trace the source of the material and apprehend the responsible parties. By contrast, acts of bioterrorism usually proceed much slower. After exposure to the biological agent, the microorganism has to grow and spread in its targets. Then there is an incubation period during which the disease develops before symptoms manifest and an accurate diagnosis can be made. In most cases, it could be days or even weeks before the dangerous nature of the circumstances is detected and the deliberate intent realized. By that time, the perpetrators could have long made their escape and covered their trails. To complicate things, many of the symptoms, especially in the initial stages of an infectious disease attack, are indistinguishable from those caused by common natural infectious diseases, such as the common cold or flu. Therefore, a bioterrorism attack may not be readily recognized. This happened in the initial phases of the 2001 anthrax attacks, where some of the physicians were not prepared to recognize the specific symptoms of inhalation anthrax and so thought the patients simply had the flu and sent them back home. It was not until after the first couple of confirmed anthrax cases were made public that physicians began checking incoming patients with suspect symptoms for anthrax exposure.

Tracing the Source

The identity of the biological agent used in a potential bioterror act is not necessarily helpful in determining the possible origin of the material. For instance, most biological weapons manufacturers and biomedical research laboratories use the same potent Ames strain of *B. anthracis*. Indeed, whole genome sequencing of the bacteria isolated from the different letters used in the 2001 mailings revealed that the strains were completely identical to each other as well as to Ames strains from many other sources, making it challenging to trace the source of the material used in the mailings. Moreover, the methods and formulations used for suspending and mixing the *B. anthracis* spores into a fine powdery substance (usually a silica-based matrix) to optimize their aerosol dispersal properties are also common among bioweapons laboratories and so also provided little information about the original source of the material. If tracing the source of the infectious agent is difficult for cases where powdery spores are available to analyze, imagine how difficult it might be for cases where the infectious agent is an infected human or animal.

Lessons Learned

The threat of bioterrorism and biowarfare is now a fact of modern life, and the events of 2001 only served to confirm this and bring out a number of glaring deficiencies in our current knowledge about microbial pathogenesis (in terms of both the microorganisms and host immunity), response measures, science education, information dissemination, and public policies regarding such acts. The public response to the spread of anthrax spores through the U.S. postal service was particularly illuminating. Whereas the postal workers, who were at highest risk, handled the situation with remarkable calm and courage, many in the general public, including policymakers, reacted just as the terrorists intended—with confusion and fear. Of course, it did not help that in the early stages of the crisis, individuals (including scientists and other experts) with a penchant for horrific scenarios dominated the media reports, while at the same time, politicians and other authorities issued contradictory statements, thereby causing confusion. Consequently, there was considerable hype and propagation of misinformation (for an example, see Box 20-1).

Contributing to this turmoil was the lack of an official, organized network of national and local scientific experts that could readily be called upon to provide accurate, unbiased, yet comprehensive and understandable information to the media and public. Clearly evident was a significant deficiency in scientific literacy, which led to misinterpretation or misunderstanding of the biological threat and nonproductive overreaction to the situation. The media, policymakers, government officials, and the general public were, and to a large extent still are, grossly undereducated in understanding science topics and associated biomedical issues, the nature of scientific endeavor, and how to cope with imposed

Box 20-1.

The Consequences of Propagation of Misinformation: The Ames Strain of *Bacillus anthracis* was not from Ames, Iowa!

Because of its high virulence, researchers use the Ames strain of *Bacillus anthracis* as a "gold standard" positive control for developing and testing efficacy of vaccines and therapeutics against anthrax. When the Ames strain was officially linked to the anthrax strain used in the 2001 anthrax mailings, everyone assumed that the strain came from Ames, Iowa.

In fact, the Ames strain was isolated from a diseased cow that died in Texas in 1981. The strain was acquired by researchers at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) in Fort Detrick, Maryland, as part of a national search for novel types of anthrax to use in testing vaccines. The army researchers at the time mistakenly believed the strain came from Ames, Iowa, because the label on the standard USDA shipping container bore the return address of the USDA's National Veterinary Services Laboratories, stationed in Ames, Iowa. When the USAMRIID scientists published a paper describing their research involving the strain, they mislabeled the specimen as the Ames strain, and the name stuck. But during investigations after the anthrax attacks, university and USDA officials in Iowa could find no record of anthrax outbreaks among Iowa cattle in years prior to 1981 or of anthrax strains being sent to the Army.

The unfortunate consequence of this misinformation was that when administration officials at Iowa State University at Ames learned of the possible connection, they ordered destruction of the entire collection of their *B. anthracis* stocks, a considerable loss of valuable scientific resource material.

Source:

Warrick J. Jan 29, 2002. One anthrax answer: Ames strain not from Iowa. *Washington Post.*

health threats, whether intentional or not. For example, many people at the time had no idea what a spore is (this is clearly not the case any longer). In fact, at the time many people, including some officials, referred to the anthrax spores as the anthrax virus. Use of the term "anthrax virus" immediately brought to mind increased fear by giving the deadly anthrax spores the properties of a virus, i.e., a biological agent that is potentially lethal, highly contagious, rapidly spread from person to person, and not treatable with antibiotics—none of which are true for anthrax (while it is deadly, it is not contagious, and it can be treated with antibiotics at early stages of infection).

It was also apparent that public officials were not consulting with unbiased expert scientists who were most familiar with the properties of anthrax spores, or they would have realized the impact that a few letters with spore-containing powder could have on postal workers and the general public. Anthrax spores are only a few microns in diameter, whereas the pores in the paper used to make envelopes are about 100 microns in diameter and so could easily allow dispersal of the envelope's contents, especially when smashed through the letter-sorting instruments at a mail facility.

Another issue that came up in the aftermath of the 2001 anthrax attacks was how far off previous predictions made by military strategists were about the circumstances and consequences of such an attack. Most pre-2001 scenarios projected large-scale exposures with catastrophic outcomes, hundreds to thousands of casualties, total collapse of overwhelmed healthcare infrastructures, and widespread ensuing pandemonium. What actually happened was far from that. The 2001 attack showed that a highly disruptive, limited anthrax attack was possible. Once it was recognized that the biological agent was anthrax and that transmission was through the postal system, the situation was relatively quickly brought under control with minimal casualties and no collapse, but rather a bolstering of healthcare infrastructure. However, there was pandemonium, although briefer and a different sort than what was projected. There was indeed a media frenzy that generated public fear and, in many cases, knee-jerk responses by public officials and policymakers. However, following a short initial period of confusion and inaction, more organized, clear, and accurate information was disseminated, and the situation dramatically improved (see Box 20-2 for an insightful anecdotal account of crisis management at a local level).

Since the anthrax event, drastic changes have occurred in how such crises are handled. One of the most valuable lessons learned was the need to provide clear and accurate information and to make everyone knowledgeable and aware of the scientific, medical, and security issues involved in preventing similar situations from occurring again and in knowing what to do if they should occur. It is hard to imagine nowadays that anyone would not notice and report a person spraying a bunch of powder in an urban neighborhood or a crowded venue or would fail to question a colleague acting secretively in a laboratory that works with infectious agents. In fact, most people have become quite sensitive to suspicious behavior, and there is an overall heightened awareness of conditions that might be favorable for potential bioterror acts. For example, there is much more scrutiny and higher

Box 20-2.

Talking to Postal Workers—and Others. . .

Although the University of Illinois at Urbana-Champaign is located in the Midwest and proved not to be a target of the bioterrorists, local postal workers, healthcare workers, and law enforcement officials were nonetheless concerned about what risk to them and the public a "suspicious powder" might pose. Immediately after the first anthrax cases became publicized, these folks, as first responders, were at the frontlines of any potential biothreats that might surface but were not prepared to deal with that threat. What the postal workers and other first responders wanted, and indeed were entitled to, was information—information that they were not receiving. This was not due to their supervisors withholding that information from them but because no one really had the expertise or experience to deal with such a situation, so there were no guidelines in place to assist them.

To remedy this, the postal workers and other first responders turned to a reliable information source that they knew they could count on for help, namely faculty members at the University of Illinois. When they contacted the Department of Microbiology, asking for experts who could talk to them about anthrax, two of the faculty responded to this request. In short order, while one of the professors visited with postal workers at many local post offices, the other professor attended meetings with area mailroom personnel, secretaries, fire fighters, and law enforcement workers. At each information gathering, the professors provided a brief explanation of what a bacterium is, what a spore is, what the disease anthrax is, and why antibiotics work in the initial stages of the disease but not in later stages. This was followed by a question and answer session open to the audience. What was most impressive about these sessions was the serious and intense nature of the interactions. Clearly, the workers had been absorbing and assimilating lots of information from various media sources because the questions asked showed very critical and logical thinking about various aspects of the crisis. Similar scenarios played out at numerous universities across the country.

The involvement of university faculty extended even further. For example, Professor Brenda A. Wilson (one of the authors of this textbook), who was teaching a course on bacterial pathogenesis at the time and was faculty advisor for the undergraduate Microbiology Club (a student chapter of the American Society for Microbiology), suggested that since no one else seemed to be providing information for the University of Illinois staff and the local community, perhaps the club members could fill that void. These undergraduate students jumped into action and organized and convened the first of what turned out to be a series of information panels on anthrax open to the public. The University administration was quite impressed with the outcome of the first student-run panel, which received a lot of local press coverage and was attended by hundreds of grateful university and community members seeking more information, so much so that the administration quickly sponsored a number of subsequent panel sessions. Indeed, this experience paved the path toward a noticeable change in local university and public health policy, so that in subsequent potential crisis situations, such as the SARS pandemic of 2003 and the more recent avian and swine flus and Ebola and Zika pandemics, the University administration, along with local health officials, was very responsive to sponsoring public information forums.

Could it be that while stumbling around trying to figure out how to help during the crisis, we may have hit upon the solution to the best response for such bioterror incidents in the future? Whether the biothreat is the result of a deliberate act or natural causes, clearly an information blitz by local experts who make the effort to communicate with the affected parties in understandable terms instead of jargon was the key to alleviating much of the fear and confusion. As a result, the public workers gained more confidence in their ability to respond to the crisis in a calm and rational manner.

Similar to what happened at the University of Illinois, professors everywhere quickly began working with undergraduate, graduate, and medical school students, who were energized to learn more about the scientific and medical aspects of anthrax, as well as other potential bioterror agents. Many professors realized that they needed to not only expand their microbiology and microbial pathogenesis courses to include sections on select agents that were not covered very well before, but also include topics of biosafety, food safety, regulatory and health policy, bioethics and socioeconomic impacts, and crisis management. The topics of biodefense and biosecurity have since been added as key topics in the microbiology, biotechnology, public health, and policy curricula at most academic institutions.

Funding from national agencies soon became available, followed by a major mobilization of resources for academic, research, and biotechnology institutions to construct and establish CDC-approved, select-agent laboratories for work on biodefense projects, as well as the necessary training to safely conduct those experiments. The process of setting up these types of laboratories also involved increased communication among university officials and the surrounding communities about the types of work that would take place and the security and safeguards of such facilities. By necessity, academic universities, research institutes, biotechnological and pharmaceutical companies, and military and government agencies have forged joint efforts toward developing training programs and policies specifically geared toward grappling with biosecurity (and biodefense) issues nationally and internationally. But, importantly, dramatic changes were implemented with regard to public communication networks. There is now a wealth of reliable, up-to-date information, response protocols, and travel advisories, available through the CDC, NIH, and other government websites, which was previously lacking.

reporting requirements of requests to obtain and work with any infectious agent, whether or not it is a potential bioterrorism agent. Editors of journal articles must now scrutinize whether submitted articles pose any potential threat that needs to be examined further. Institutional biosafety committees at universities and companies are charged with reviewing and approving in advance all proposed research with any pathogen or toxin for compliance with strict regulations stipulated by government agencies, such as the CDC, the NIH, and the U.S. Department of Agriculture (USDA). Moreover, detailed policies, medical infrastructure, emergency response and crisis management plans, and communications networks are now in place and stand ready to be implemented when needed.

The "Top Four" Bioterror Agents

Biowarfare specialists, and presumably bioterrorists, have chosen a limited number of preferred biological agents for development as bioweapons (the so-called "select agents" listed in Table 20-2). Of these, we will consider here four examples that stand out among the others: *B. anthracis* spores, plague-causing *Y. pestis*, smallpox virus, and *C. botulinum* neurotoxin serotype A (BoNT/A). We will briefly explore some of the reasons why these four examples might be among the top choices for use as bioterror agents, as well as some of the limitations for why they might currently be less of a threat than previously thought.

Bacillus anthracis Spores

Before 2001, anthrax spores had often been touted as the top choice in bioweapons for biowarfare, or "germ warfare." The events of 2001 only confirmed this claim, especially when the spores used were found to be of extremely high quality and potency, so-called "weapons-grade." Anthrax spores are one of the easiest of the bioterror agents to manufacture, manipulate, and develop as a bioweapon. They can be produced in large quantities using basic knowledge of microbiology. Naturally produced spores usually tend to clump together and are not very infectious due to the clumps being too big to pass easily through air passages in the lung. However, anthrax spores can be further refined. With just a little bit more effort and resources, the spores can be coated with silica or other matrices and the coated spores then ground into a fine powder for aerosol dispersal. The resulting spores can be spread readily by high-tech delivery vehicles, such as missiles, aerial bombs, crop dusters, or sprayers, or even by low-tech methods, such as mail envelopes.

Anthrax spores also have other properties that make them ideal for use as a bioweapon. They are very hardy and highly heat-resistant, and they can remain viable in the soil, even with exposure to extreme environmental conditions, for many years. This means that they can be stockpiled in dry form for decades without losing their viability or potency. It also means that they are difficult to destroy, making exposed areas difficult to decontaminate. As a case in point, it took over three months and more than $23 million to decontaminate the Hart Senate Building after an envelope mailed to a senator was opened in October of 2001 and found to contain anthrax powder.

Anthrax spores are hard to detect—any unknown powder that might be discovered most likely consists of the matrix and may not even contain any spores. Indeed, there were numerous "false alarms" reported during the period of the anthrax attacks that upon closer inspection turned out to be just talcum powder, chalk, or powdered sugar. There is also no immediate indication of exposure. There is no obvious cloud, except for massive exposures of fine powder, no color, no smell, and no taste. There is a delay period before onset of symptoms. Add to this the fact that the initial symptoms are fairly nonspecific and resemble the common cold or flu, the chances diminish that a doctor will accurately diagnose and treat the disease within the critical window when treatment could still be effective. Unfortunately, once symptoms manifest, the likelihood of survival plummets drastically, even with antibiotic treatment, because as we saw in chapter 12, anthrax disease is largely mediated by anthrax toxin. Because anthrax disease progression after onset is very rapid in the case of inhalation exposure, anthrax is almost always fatal if not treated early.

The least serious form of anthrax is cutaneous (skin) anthrax (commonly known as "wool-sorters disease," because people working in tanneries with wool and other animal hides frequently contract it). In cutaneous anthrax, the spores enter the body through localized cuts, abrasions, or other wounds and germinate at the infection site and cause edema (swelling) and cell death, forming a characteristic black-centered lesion (called an "eschar"). The death rate for untreated cutaneous anthrax is about 20%, but because it is localized and does not spread rapidly, it is readily treatable with antibiotics. All of the 2001 victims that contracted cutaneous anthrax, including a 7-month-old toddler, were treated successfully with antibiotics (ciprofloxacin) and survived. Another form of anthrax is intestinal anthrax caused by consumption of contaminated, undercooked meat. The spores germinate in the intestine and cause acute intestinal inflammation, nausea, abdominal pain, and severe bloody diarrhea. Intestinal anthrax results in death in about 60% of untreated cases due to the disease progressing to the systemic form.

The most lethal forms of anthrax are the systemic and inhalation forms, with the victim usually succumbing to a rapid onset of massive hypotension, generalized (especially pulmonary) edema, massive bacteremia (with blood bacterial concentrations reaching $>10^8$ CFUs per mL of blood), encephalitis, and acute, fatal toxic shock. While the bacteremia can be treated with antibiotics at early stages, there is a point of no return when the disease switches over to a toxin-mediated disease (see chapter 12 for details on the action of anthrax edema and lethal toxins); by then there is little hope for survival since there are no known antidotes available against the toxin effects. Death rapidly ensues. Administering antitoxin, in the form of antibodies against the edema and lethal toxins, has some benefit for treatment of cutaneous anthrax, but it has not been very successful for inhalation anthrax, presumably because by the time symptoms appear, the disease has progressed too far for the antitoxin to reverse the inflammation and toxic shock.

With all these advantages for using anthrax spores as a bioweapon, the only notable weakness is that while it is lethal for the individual who received the initial dose of inhaled spores, it is not transmissible from person to person. While not ideal for the goals of a bioterrorist, this is good for us, because unlike a highly contagious disease like smallpox (see next section), spread of the illness among a population can be brought rapidly under control and, once identified, protective measures, such as use of breathing masks and administration of prophylactic antibiotics, can be imposed. The other thing to remember about anthrax is that the spores have to enter the body in order to germinate and cause disease. This means they can be washed away from the body and other surfaces. It is amazing what a Band-Aid can do to prevent cutaneous anthrax due to a cut or abrasion.

There is an approved vaccine available against anthrax. The efficacy, safety, and production standards of the anthrax vaccine are considered to be acceptable, except for pregnant women and immunocompromised individuals, and the vaccine is routinely administered to the members of the U.S. armed forces and other persons at risk for anthrax exposure. One problem with this vaccine is the need for yearly boosters to maintain efficacy, but other alternative anthrax vaccines are under development.

Smallpox

Although smallpox is caused by a virus, we include it here as a contrast to bacteria-based biothreats and because of its known development as a bioweapon in the past. Indeed, smallpox has been touted as a "good" bioterror agent due to its lethal and extremely contagious nature, the long incubation time before characteristic symptoms appear, and its viral nature that makes it difficult to treat (i.e., it cannot be treated with antibiotics). One bioterror scenario often put forth is where contaminated material, such as the blankets and bed linens given to the Native American Indians mentioned earlier, could be distributed to the public through commercial retailers. And, because of the long incubation period and its highly contagious nature, potentially hundreds of households might be infected before the disease is recognized and measures for containment and treatment mobilized.

Another scenario often cited is one where a suicidal bioterrorist might serve as an incubator by self-inoculation and then would go about spreading the disease through contact with others. Since there is a long lag period before symptoms manifest, the bioterrorist could in theory come into contact with tens to hundreds of people during that period (imagine the person going to a stadium-packed ballgame, a large concert, or even a crowded nightclub). And, since smallpox has been eradicated and no one has been vaccinated since the 1970s, the highly contagious, deadly disease could spread rapidly through the younger members of a population. Years before smallpox was eradicated, such an incident actually did happen accidentally in the United States in 1947, when a U.S. citizen, who had traveled to Mexico and contracted smallpox, re-entered the United States and traveled by bus to New York City, where he then circulated for a number of days before being hospitalized and diagnosed with smallpox, and eventually succumbing to the disease. Contrary to what you might expect, there were no massive outbreaks and thousands of New Yorkers did not contract the disease and die. Instead, health officials and local authorities swiftly mobilized, isolating the infected man and all the people with whom he had come in contact, quarantining and vaccinating them. They then set about vaccinating millions of New Yorkers over the course of about two weeks, effectively containing the outbreak within three weeks. Consequently, the number of actual smallpox cases (12) and deaths (2) was relatively small, considering what could have happened without the massive isolation and vaccination campaign that was mobilized.

Key to the successful containment of this outbreak, as well as others that have occurred and any that might occur in the future, was the ability to vaccinate everyone who had been exposed but had not yet manifested disease. This was possible because of the long incubation period during which vaccination is still effective in preventing disease. If an exposed person is vaccinated within a window of 3–4 days after exposure, the disease is either completely prevented or greatly ameliorated. The critical factor then becomes ensuring that all individuals who may have been exposed to the infected person are indeed identified, quarantined, and vaccinated. Today, this is quite feasible through modern, sophisticated contact-tracing capabilities and epidemiological resources that authorities have at their disposal. It is also possible to get information out quickly through the Internet using new informal media channels, such as Facebook and Twitter.

Yersinia pestis

The flea-borne bacterium *Y. pestis* owes its fear factor primarily to our collective memory of the horrific plagues (the "black death") of the Middle Ages, which decimated entire populations at the time. The main aspect that dampens the effectiveness of using plague as a bioterror weapon is the requirement of the flea as a vehicle. Prevention of transmission by controlling the insect vector is a very effective defense strategy for bubonic (flea-borne) plague. Nevertheless, during World War II, the notorious Unit 731 of the Japanese imperial army experimented with several bioterrorism agents, including *Vibrio cholerae* and *Y. pestis*, on unsuspecting Chinese populations. Fleas infected with *Y. pestis* were disseminated by spraying and in bombs dropped from airplanes. It is not clear how successful these efforts were since records are sketchy, but the purported number of victims is high.

Another aspect that diminishes plague as an effective bioterror agent is its sensitivity to antibiotics. For instance, there were periodic outbreaks of bubonic plague in Vietnam during the Vietnam War, which were readily controlled with administration of antibiotics. Although still endemic in certain parts of the world, *Y. pestis* is a rare cause of disease today in the United States and other developed countries, infecting perhaps a few hunters or hikers each year that inadvertently come in contact with animals carrying infected fleas. Those individuals who do become infected are usually diagnosed and treated effectively with antibiotics.

The current fear most often cited about use of *Y. pestis* as a bioterror agent is the possibility that the terrorists might render the bacterium resistant to multiple antibiotics and deliver it via aerosol spray. Despite major efforts to understand pneumonic (pulmonary) plague, which is spread directly from person-to-person via inhalation without using the flea as a vehicle, the fact remains that scientists still do not know how to simulate well this most lethal and contagious form of plague or how to effectively treat it, particularly if it is resistant to multiple antibiotics. Still in our favor is the fact that the fitness toll, which would have to be overcome to develop a strain that is resistant to all available antibiotics, diminishes the likelihood that a strain resistant to all antibiotics could be developed. Nonetheless, there have been reports of naturally occurring multidrug-resistant *Y. pestis*, so it is conceivable that a viable organism with more than a few resistance genes could be constructed. But even if such as strain were developed and used, there are still alternative treatment modalities available, including passive immunization and vaccination. And, as we saw in chapter 16, we are learning to deal with the escalating threat of multidrug-resistant microbes of both the natural and man-made varieties. Unfortunately, only one vaccine against *Y. pestis* has been developed, but it is no longer approved in the United States because of poor efficacy and issues with side effects. Similar to the case with *B. anthracis*¸ an effective, safe vaccine that could be used in the general population would largely eliminate *Y. pestis* as a bioterror threat. Several new plague component vaccines, comprised of *Y. pestis* F1 and V antigens, are currently being developed, but none have been licensed yet.

Botulinum Neurotoxin

Although botulinum neurotoxin is more properly defined as a chemical terror agent since it is a protein rather than a bacterium or virus, it is often included in the bioterrorist's repertoire of select agents. The main fear here is that it might be introduced into water or food supplies. Add the fact that it is widely available for biomedical and cosmetic applications (see chapter 12), then the likelihood that it could be acquired and used to poison vulnerable water and food supplies is not too farfetched.

Many experts discount water supplies as a viable route of delivery because of the dilution factor, which certainly is true for the less-stable purified form of the toxin. However, what many do not realize is that the native clostridial neurotoxins are produced naturally from the bacteria as complexes with chaperone proteins that make them much more heat-resistant and stable to acid conditions (such as that encountered during passage through the stomach) (see Figure 12-16). For example, the juice from a jar of spoiled string beans contaminated with botulinum neurotoxin dumped into a juice dispenser or a small water supply could have devastating consequences. Even so, there is little doubt that the more vulnerable, centralized milk or food supply chains are generally viewed as being of greater concern. In-deed, there already are numerous incidents of natural or accidental food-borne botulism reported annually due to undercooked or unpasteurized food and juices. Of course, an obvious preventive measure that could be taken is to bet-ter educate people regarding the importance of thoroughly cooking their food—an approach that has already had some, albeit not complete, success with other food-borne illnesses (see later sections).

An advantage of using botulinum neurotoxin as a bioweapon is the difficulty in detection and diagnosis before the onset of disease symptoms. The extreme potency of the toxin makes the limits of detection challenging to reach. Unlike a bacterium or virus that can be propagated through growth or whose DNA/RNA can be amplified by PCR methods to increase the likelihood of detection, botulinum neurotoxins act at picomolar (10^{-12} mole per L) or lower levels, do not replicate, and are proteins (not DNA or RNA). In addition, symptoms can take anywhere from a few hours to several days to appear. These factors make detection, diagnosis, monitoring, and tracing of the source very difficult.

The biggest concern about an outbreak of botulism is actually logistical in nature, namely the lack of sufficient numbers of available ventilators in local hospitals. As detailed in chapter 12, once a person has been intoxicated with botulinum neurotoxin and symptoms of paralysis have manifested, there is no reversal of the paralysis possible since there are no post-exposure antidotes available. This means that even if a botulism victim receives antitoxin to prevent any further intoxication, that person is still paralyzed and could remain so for up to 4–6 months or longer, depending on the toxin serotype used and the total dose received before antitoxin was administered. The only treatment available for the paralyzed patient is supportive, primarily in the form of a mechanical ventilator to breathe. As per current practice, most hospitals run at about 95% capacity with regard to the availability of respiratory ventilators since they are expensive instruments and are also needed for many other medical purposes. So, should a moderate to large outbreak of botulism occur, this would greatly tax the existing infrastructural capacity of nearby hospitals to handle such an acute, long-lasting demand—truly a logistical nightmare.

What If Bioterrorists Hit Us with Something Completely New?

Could an inventive, scientifically trained bioterrorist develop, steal, or otherwise procure a biological agent with such new or unusual properties that it could not be initially identified or treated? While it is impossible to know for sure if such a scenario will occur, with the advanced biotechnology capabilities currently available, many researchers and policymakers fear this is a real possibility, particularly given current advances in synthetic biology, genomics, and biotechnology in light of worrisome political trends. Fortunately, we now are readier than ever to meet such a threat head-on. The highly sophisticated genomic, analytical, and detection technologies that are currently available (see chapters 5 and 9) have already proven time and again to be able to rapidly and accurately identify the microorganisms and toxins responsible for various disease outbreaks. It is now quite feasible to obtain within days the complete genome sequence of any unknown bacterium, fungus, virus, or protozoal parasite isolated from an encounter. The surveillance and response networks now in place are highly sophisticated and well established to monitor work with select agents and other pathogens.

Clear examples of the effective mobilization of these sorts of efforts occurred during the SARS pandemic of 2002–2003 that followed the anthrax attacks and the more recent pandemics of Ebola in 2014–2015 and Zika in 2016–2017, which were caused by viruses previously unseen in the human population. In addition, mobilization efforts have effectively dealt with more recent avian (H_5N_1) and swine (H_1N_1) flu pandemics and numerous food-borne *E. coli* and *Salmonella* outbreaks, which involved unusual variants of known viral and bacterial pathogens, respectively. In each case, the culprit was rapidly identified, sequenced, compared to other pathogens in the databases, and new PCR-based or enzyme-linked immunosorbent assay (ELISA)-based diagnostics were quickly developed to detect it, and at least in the case of viruses, new vaccines were developed to prevent its spread. Besides next-generation sequencing technologies for studying bacterial threats, there are also high-throughput and multiplex technologies in place to screen for potential antibiotics or therapeutic drugs and to identify resistances and potential vaccine targets. Based on these precedents, there is little doubt that the power of genomics and all of its associated enabling capabilities could be quickly ramped up for any newly encountered biothreat in the future. Nevertheless, constant vigilance and the biosecurity measures discussed in the next section are essential in this era of bioterrorism, for, whether deliberate or not, the biothreat is real, and microbiologists will be in the frontlines combating it.

Biosecurity in a Complex, Dynamic, and Ever-Changing World

While the anthrax attacks of 2001 made biodefense a major health priority in the United States, the recent pandemics of the SARS, avian and swine flus, Ebola and Zika that followed in its wake, the growing number of food-borne illnesses, the alarming spread of multidrug-resistant pathogens, especially bacteria as summarized by the CDC in 2013, and the persistence or emergence (or re-emergence) of deadly infectious diseases have now converged to make **biosecurity**, which includes biodefense, the most important global health priority. The human cost alone is staggering (Table 20-3) and cannot be ignored.

Biosecurity is a much more encompassing concept than biodefense alone and entails putting into place a complete set of preventive and containment measures intended to eliminate or reduce a wide spectrum of potential biological risks and threats, ranging from natural or accidental exposures from infectious agents or toxins to deliberate acts of bioterrorism or biowarfare. Many diverse factors impinge on global biosecurity, impacting the severity and urgency of the biothreat event and our ability to quickly and effectively respond to and recover from it. Biosecurity is a challenging yet critical topic of concern that impacts many facets of our society today, and one should have no doubt that microbiologists have and will continue to play a starring role in this battle. But, importantly, microbiologists must work closely with health officials, government agencies, and policymakers to bridge the gaps between scientists and nonscientists, to enhance science literacy and competency, and to build the necessary infrastructure to improve biosecurity measures and responses. In the United States, the **National Science Advisory Board for Biosecurity**, comprised of representatives from across various government agencies, is tasked with recommending policies regarding how to prevent publications about biotechnology research from contributing to bioterrorism without hampering scientific progress.

As pointed out earlier, distinguishing a deliberately introduced infectious disease from a naturally occurring or newly emerging infectious disease is sometimes very difficult.

Table 20-3. The human cost of selected biological threats of concern

TYPE OF CASUALTIES	BIOLOGICAL AGENT	NO. OF ASSOCIATED INCIDENTS	NO. OF ASSOCIATED DEATHS
Bioterrorism	Ricin assassination of Georgi Markov in 1978	1	1
	Salmonella enterica serovar Typhimurium in salads in 1984 (Rajneeshee)	751 cases	0
	Sarin gas in 1995 (Aum Shinrikyo)	>2,000 hospitalized	12 total
	Anthrax mailings in 2001	22 cases total	5 total
Natural	Malaria worldwide	300–500 million cases per yr	2 million per yr
	Food-borne illnesses in United States	76 million cases/325 hospitalizations per yr	5,000–10,000 per yr
	Tuberculosis worldwide	8–10 million cases per yr	2 million per yr
	Pneumococcal (*S. pneumoniae*)-related illnesses worldwide	Millions of cases per yr	2 million per yr
	HIV/AIDS worldwide	>60 million cases total	>20 million total (500,000 in United States)
	Hepatitis C in United States	170 million total	10,000 total
	Septic/toxic shock worldwide	20 million cases per yr (100,000 cases per yr in United States)	10 million per yr (50,000 per yr in United States)
	SARS virus worldwide in 2002–2004	8,096 confirmed cases total	774 total confirmed
	Ebola outbreak 2014–2016	28,652 total cases/15,261 confirmed	11,325 total confirmed
	H_5N_1 (avian) influenza in 2003–2007	436 confirmed cases total	262 total confirmed
	H_1N_1 (swine) influenza in 2009 in United States	43–89 million cases/274,000 hospitalizations total	12,470 total confirmed
	Seasonal (mostly H_1N_1 and H_3N_2) influenza (non-2009) in United States	15–60 million cases/110,000 hospitalizations per yr	36,000–42,000 per yr

As mentioned before, due to their "dual-use" nature, building a firewall is inherently more challenging for biological agents than other types of security threats. Thus, the best biosecurity strategy encompasses both intentional and unintentional disease outbreaks. In this regard, while warning and prevention are greatly preferable to coping with the consequences of an attack, the fact remains, and we have seen ample examples of this in recent years, that it is possible for a new biothreat to emerge despite our best preventive measures. This means that it is critical for a greater emphasis to be placed on bolstering public awareness through education in addition to improving healthcare capacity and response policies. For instance, one of the most remarkably effective ways to prevent disease transmission is to practice good hygiene by frequently washing hands, covering the mouth when coughing, and avoiding direct contact (kissing, hand shaking) with others when one of the parties is clearly ill. The massive advertisement campaign launched in response to the flu threat was very effective in educating the public and mitigating the spread of disease. However, a critically important component necessary for implementing this effective preventive measure is ensuring that people everywhere have access to plentiful clean water sources and sustained sanitation.

Food Safety and Biosecurity

Each year, over 76 million people in the United States (i.e., 25% of the population) suffer from a food-borne illness. Although most of these individuals have self-limiting diarrhea or nausea and are never diagnosed, there are about 325,000 hospitalizations and 5,000 to 10,000 deaths. In the past few decades, there has been a tremendous scale-up in food production worldwide, from vast herds of cattle, large confined feedlots, and huge slaughterhouses to the many hundreds of distribution centers and supermarkets. Trade barriers have come down, and free trade has permitted food to be shipped around the world in a matter of a few hours to a few days. This globalization of food distribution also enables the rapid spread of a potentially contaminated food source. Undoubtedly, these conditions have contributed to the emergence and prevalence of food-borne illnesses as a major global health concern. Add to this the fact that over 50% of our food dollars are now spent on prepared food outside of the home, and it is not hard to imagine the potential devastating consequences a global biothreat might pose to this situation.

The complexity of the modern food preparation and distribution process makes epidemiological tracking of the sources of contamination challenging, although there have been noticeable advances. Several federal agencies have set up networks, such as the Foodborne Diseases Active Surveillance Network (FoodNet) and the Food Emergency Response Network (FERN), to address this critical need. The U.S. Food and Drug Administration (FDA) has sponsored a **hazard analysis and critical control point (HACCP)** program that monitors food distribution at critical control points where contamination is most likely to occur. The USDA has established a similar assessment method for identifying the most vulnerable target sites for introduction of contamination within the food-processing system, called **CARVER+Shock** (for criticality, accessibility, recuperability, vulnerability, effect, recognizability, and shock effect). However, despite these recent efforts to enhance surveillance, monitoring, reporting, containment, and recovery from food-borne illnesses, the increasing number of high-profile incidents, which have largely been caused by unintentional contamination, amply illustrates the widespread havoc, alarm, and economic consequences that can result from an outbreak of contaminated food. The greatest problem with current efforts to ensure food safety is that they are focused on too broad and too vast a target: the entire food processing chain—from field to table. There are simply too many spots along the way from the farm to the dinner table for a contaminated food sample to make it to the consumer, sometimes with lethal outcome, and when such a slip occurs, consumer confidence in the food industry rapidly plummets, with substantial economic impact.

The real question that emerges from this situation is: Exactly what level of food safety do consumers want the agricultural and food industries to achieve and the government to enforce? In recent years, considerable effort by industry and policymakers alike has been spent grappling with this urgent need. So far, most proposed measures have been aimed at further enhancing the measures already in place, such as those mentioned previously, and have been reinforced by new legislation, such as the Food Safety Modernization Act of 2011, which shifted the emphasis of food safety regulations from responding to contamination to preventing it. However, while these efforts have helped and many of the proposed measures implemented, these actions cannot possibly ensure the desired near-complete protection from an incident.

The Case for Food Irradiation

The proposed use of **food irradiation technologies** to enhance food defense capabilities has emerged as a topic of intense current interest. The premise is that food irradiation technology, which can be applied at the very last stage of the food processing chain, could achieve the near 100% contamination-free status of the food products that consumers are demanding. The problem with its implementation is that, for most consumers, the idea of food irradiation is a "scary" technology because it invokes fear of radiation poisoning and loss of the food's taste and nutritional quality. The initial upfront costs associated with deploying this technology are also higher than most members of the food industry are willing to invest, meaning that there will have to be substantial governmental subsidies to encourage compliance.

Food irradiation technology, also called "cold pasteurization" or "cold sterilization," can eliminate disease-causing microbes from foods by treating the food with approved levels of ionizing (high-energy) radiation. There are three types of irradiation used: gamma rays, electron beams, and X rays. **Gamma rays** are high-energy photons produced by radioactive material (usually cobalt-60 or cesium-137) that can penetrate food and other materials. Contrary to popular belief, they do not make the target material radioactive. Currently, this method is used to sterilize medical, dental, and household products, especially those used for infants or patients that are immunocompromised, such as those undergoing cancer treatment (Table 20-4). **Electron beams** are streams of high-energy electrons produced by an electron gun that can penetrate materials to a depth of about 3 cm. There is no radioactive material involved in generating electron beams. This technology has been used for over 20 years to sterilize medical devices and other household products and is gaining application for food irradiation. **X rays** are generated when an electron beam is directed at a thin plate of gold or other metal. The X rays produced by this technology are more powerful than those used for medical (e.g., chest X rays) or dental imaging. Again, there is no radioactive material involved, and the beams can penetrate foods to a depth of about 38 cm. X-ray sterilization technology is still in development and is not currently being used commercially.

These forms of radiation are ionizing in that they cause loss of electrons from biological molecules and from water. DNA is the largest target molecule in the cells of contaminating organisms, and DNA damage is lethal to bacteria, parasites (e.g., worms), and insects present in food. Ionization can directly rupture chemical bonds in DNA, leading to damage that often cannot be repaired. In addition, ionizing radiation converts water into highly reactive, but short-lived, hydroxyl radical (•OH) that reacts with the backbone and bases of DNA causing further damage. Unrepaired DNA damage blocks the replication and growth of contaminating

Table 20-4. **History of food irradiation technology use**

YR APPROVED	FOOD	DOSE (kGy) PERMITTED	PURPOSE
1963	Wheat flour	0.2–0.5	Control of mold
1964	White potatoes	0.05–0.15	Inhibit sprouting
1986	Pork	0.3–1.0	Kill *Trichia* parasites
1986	Fruits and vegetables	1.0	Insect (fruit fly) control, increase shelf life
1986	Herbs and spices	30.0	Sterilization
1990 (FDA)	Poultry	3.0	Bacterial pathogen reduction
1992 (USDA)	Poultry	1.5–3.0	Bacterial pathogen reduction
1997 (FDA)	Red meat	4.5	Bacterial pathogen reduction
1999 (USDA)	Red meat	4.5	Bacterial pathogen reduction
2008 (FDA)	Lettuce and spinach	4.0	Bacterial pathogen reduction, increased shelf life

organisms, resulting in cell death. Because DNA is the major target for killing organisms by ionizing radiation, genome size is a factor in how readily an organism is killed by irradiation processes (Box 20-3). Although effective against bacteria, parasites, and insects, much higher doses of ionizing radiation are required to kill bacterial spores, which resist many kinds of sterilization methods, and viruses, which have small-sized genomes of nucleic acids tightly compacted in a protein coat. Ionizing radiation and hydroxyl radicals can damage proteins, such as toxins and prion proteins, but protein inactivation is not the major mechanism by which irradiation kills organisms, spores, and viruses. Finally, some extremophilic bacteria species, such as *Deinococcus radiodurans*, can survive high doses of ionizing radiation and dehydration. In fact, *D. radiodurans* was initially isolated from a tin of canned meat (i.e., "Spam") that had been exposed to high doses of gamma irradiation to sterilize it. Indeed, because of its high resistance to radiation, *D. radiodurans* has been genetically engineered for bioremediation of chemical wastes containing radioactive materials. Fortunately, *D. radiodurans* is not a contaminant in the normal food chain, keeping food irradiation as a viable method for food sterilization.

The interesting thing about irradiation technology is that despite public fear, it is already being widely used by consumers. Most medical devices, Band-Aids and other wound care products, cosmetics, diapers, and infant care products, as well as feminine products, are already routinely treated with irradiation. Many food products have also been irradiated to kill insects, to prevent sprout growth, and to prevent spoilage (Table 20-4). Irradiated food generally has a longer shelf life, does not spoil as readily, and in some cases, has enhanced antioxidant capacity.

Box 20-3.

How Do You Measure Food Irradiation?

The dose of irradiation is measured in Grays (Gy), i.e., the amount of energy transferred to the food, microbe, or other substance, 1 Gy = 1 joule/kg.

One chest X ray = 0.5 mGy
One abdominal + pelvic CT scan = 30 mGy
Dose to kill 10^4–10^6 *Salmonella* bacteria in a piece of chicken ~4.5 kGy, in juice ~3 kGy
Dose to kill 10^4–10^6 *Listeria* bacteria in pork ~2 kGy at 0–10°C, ~3 kGy at 16°C

The killing effect of irradiation on microbes is measured in D-values. An irradiation D-value of 1 will kill 90% of the microbes present. An irradiation D-value of 2 will kill 99% of the microbes present. The size of the DNA of an organism is a factor in how readily that organism is killed by the irradiation:

Parasites and insects—rapidly killed at D-values < 0.1 kGy
Bacteria—killed at D-values = 0.3–0.7 kGy
Bacterial spores—killed at D-values ~2.8 kGy

(Note: During the 2001 anthrax attacks, mail was decontaminated with ~30 kGy.)

Viruses—killed at D-values >10 kGy
Prions and toxins (peptides, proteins)—not affected by irradiation

(Note: NASA routinely sterilizes food for astronauts with 42–44 kGy of irradiation.)

The most often cited concerns regarding food irradiation technology are its potential harmful effects on food quality and the consumer. Extensive longitudinal feeding studies (since the 1950s) have revealed no significant ill effects on animals (mice, rats, dogs, etc.) or humans (hospital patients and astronauts). As the high-energy beam is absorbed, food warms up, with a dose of ~10 kGy warming the food by ~2.5°C (most food irradiation involves doses of less than 4 kGy) (Table 20-4). This warming can result in some treated foods tasting slightly different or, in the case of leafy vegetables, wilting a little. If food still has living cells (e.g., seeds, shellfish, potatoes, and eggs), then those cells will be killed, just as the microbes are, and this may cause the food to taste a little different. At levels approved for use on foods, some vitamins (e.g., thiamine) are slightly reduced, but most fatty acid, amino acid, and other vitamin content is not significantly altered. Irradiated foods, especially meat containing fat, can have slightly higher amounts of 2-alkyl cyclobutanones (derivatives of triglycerides), which could cause DNA damage, but these compounds are apparently not readily metabolized by animals and are usually excreted. Overall, despite these minor potential concerns, the conclusions of most scientists and government food safety agencies are that food irradiation technologies are generally safe and highly effective in decontaminating food, so be prepared to see more and more use of this technology.

The Future of Biosecurity

Key to an effective biosecurity strategy will be a better understanding of the driving forces that are important for transmission and perpetuation of infectious diseases and then implementation and management of effective preventive, treatment, and containment measures. To develop better predictive models for disease outbreaks and spread, policymakers will need to increase support for research efforts to understand microbial pathogenesis and the driving forces behind pathogen evolution and ecology. We also need more support for research efforts to develop therapeutics and alternative treatment modalities for multidrug-resistant microbes, to treat post-exposure intoxications, and to identify and treat new diseases of unknown origin. For this to occur, it will be necessary to strengthen local, national, and international capacities to prevent and control disease outbreaks.

Unfortunately, at this writing, funding to the National Institutes of Health in the United States, one of the major drivers for this type of research, has been largely stagnant or declining in recent years, and the chances of obtaining an investigator-initiated research grant are hovering at historical lows. Moreover, while global disease surveillance systems and international cooperation have improved tremendously in recent years, they are still not nearly where they need to be in terms of resources and integration. Finally, science education will be a critical deciding factor in enhancing the confidence of policymakers and the public in science and scientists, which will move this endeavor forward for greater preparedness against natural and man-made biothreats. Considering the real biothreats that are already upon us, some of which were considered in detail in this chapter and in earlier chapters, there is a real urgency for us to move in this direction rapidly with a long-range vision.

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Questions

1. Why is anthrax a "weapon of choice" for bioterrorists? What might make it better than smallpox or plague?
2. What is the major concern about the use of botulinum neurotoxin as a bioweapon?
3. What are some of the ways that we can minimize the detrimental impact of bioterrorism on society and science?
4. How do you think the policies put in place since the 2001 anthrax event have impacted our relationships with other countries? Do you think these policies have impacted the leadership role of the United States in science and technology? If so, how?
5. How do you think microbiologists and other scientists can improve public policy and governmental infrastructure to best respond to potential biothreats?
6. What is the difference between biodefense and biosecurity?
7. Should local authorities or citizens stockpile antibiotics to protect themselves against a potential bioterror act? Explain your answer.
8. Should citizens buy gas masks to protect themselves and their families from biological agents? Why?
9. Which of the following biological agents is not contagious?
    a. Smallpox
    b. Anthrax
    c. Pneumonic plague
    d. Bubonic plague
10. Which of the following is a true statement about smallpox?
    a. A victim with smallpox is contagious.
    b. Cases of smallpox are still found occasionally in Africa.
    c. The recommended treatment for smallpox is ciprofloxacin or doxycycline.
11. Which bioterror agents will persist in the environment? Explain your answer. What steps would be necessary to clean up or live in these contaminated environments?
12. Suppose you are doing basic research on an attenuated version of a select agent. What concerns should you have about genetically manipulating these strains to contain antibiotic resistance markers?
13. How informed do you think the public is about key issues of biosecurity, including biodefense? What measures do you think should be taken to increase this knowledge?
14. What roles do science education and literacy play in biosecurity? Do you think that this is important?
15. Ionizing radiations used in food irradiation technology include
    a. accelerated electrons in electron beams.
    b. high-energy photons in gamma rays.
    c. high-energy photons in X rays.
    d. high-energy microwaves.
    e. accelerated neutron beams.
16. Food irradiation technology has the potential to address major food safety problems, such as (choose all that apply)

a. short shelf life.
b. high initial microbial loads.
c. quarantine requirement for insect pest management.
d. unsafe consumption of contaminated fresh fruits.
e. high cost of migrant workers to harvest crops.

17. An irradiation dose of 3 kGy will kill
 a. >99% of the bacteria in a piece of chicken.
 b. 90% of the bacterial spores in a cup of green beans.
 c. 90% of the viruses in a cup of water.
 d. 10% of the prions in a piece of deer meat.
 e. all of the fruit flies in a pint of blueberries.

18. Doses of less than 1 kGy of irradiation treatment have very little to no effect on physical and sensory quality attributes of most fruits and vegetables, but some reports indicate that higher doses can (choose all that apply)
 a. decrease content of some sensitive water-soluble vitamins, such as vitamins C and B1.
 b. decrease content of some fat-soluble vitamins, such as vitamins A and E.
 c. decrease carotene content.
 d. increase fruit ripening.
 e. decrease softness of fruit texture.

Solving Problems in Bacterial Pathogenesis

1. You are working as an emergency doctor at a community health clinic near a convention center. Over the course of the afternoon on the second day of a conference being held in the convention center, approximately 200 of the attendees at a conference come into your clinic with complaints about double vision, dry mouth, difficulty swallowing, drooping eyelids, and overall weakness.
 a. What is the most likely biological agent (plague, anthrax, smallpox, botulinum toxin) causing these symptoms?
 b. Considering the nature of this outbreak, what are the first steps you take as a first responder to this emergency? What is your primary concern in dealing with this crisis?
 c. By the end of the day, your worst fears are that this may be an act of bioterrorism. What are the next steps that you should take?

2. You work for the Department of Homeland Security (DHS) and have just received a call from a group of researchers at the USDA to consult on a case of unusual fatal food poisoning in Wisconsin due to contaminated cheese. Based on 16S rRNA gene analysis, the researchers identified the pathogen as *Listeria monocytogenes.* This new, highly virulent strain appears to cause symptoms of listeriosis even in previously healthy, immunocompetent individuals, including fever, headache, muscle ache, nausea, and vomiting. But then, over 1–3 days this is followed by onset of anasarca (severe edema, with widespread swelling and accumulation of fluid in all of the tissues and cavities of the body at the same time), stiff neck, disorientation, and convulsions, and in most cases death within 1–2 days of onset. Autopsies of individuals who died showed that bacteria were found throughout the body. The researchers subsequently determined that in tissue culture this highly virulent *Listeria* strain can invade epithelial and endothelial cells and can spread from cell to cell. They also found that similar disease symptoms occurred in mice fed cheese inoculated with bacteria. The researchers also discovered that injection of medium from cell cultures into mice resulted in massive edema. In your expert opinion as a microbiologist and researcher in the field of bacterial pathogenesis, is there plausible cause for the DHS to be concerned that the incident described here could be a potential act of bioterrorism? Be sure to state your rationale.

3. Again, in your capacity working for the DHS, you have just received an urgent call from the Centers for Disease Control and Prevention (CDC) to consult on another unrelated case of an unusual disease outbreak that researchers at the CDC have been investigating. The researchers isolated a new gram-positive bacterium related to *Listeria* from an outbreak of food poisoning in Wisconsin due to contaminated cheese that appears to cause painful gastritis and, in about half of exposed individuals, sudden onset of bleeding ulcers, followed by death from toxic shock within 2–3 days. Upon biopsy of infected individuals, it was found that the bacteria were growing on the surface of epithelial cells lining the gastric pit of the stomach. Autopsy of individuals that died showed that bacteria were found only in the stomach and not in any of the other body organs. The researchers at the CDC have subsequently determined that, unlike *Listeria monocytogenes*, this bacterium does not invade epithelial cells or spread from cell to cell. When the researchers grew the bacteria in laboratory medium overnight and then introduced the filtered culture medium onto the surface of the stomach epithelial layer in experimental rabbits, they found that bleeding stomach ulcers were formed and the rabbits died from toxic shock within 1–2 days. When they analyzed the filtered culture medium by SDS-PAGE, they found that five protein bands were present on the gel. The researchers cut out each of these bands from the gel and subjected the proteins to protease digestion, followed by N-terminal sequencing of the cleaved peptide fragments. When they compared the sequences of the cleaved peptides from

each protein with the known protein databases, they found that protein #1 had sequence homology to *Helicobacter pylori* urease, protein #2 had sequence homology to the mucinases of *Helicobacter pylori* and *Vibrio cholerae*, protein #3 had sequence homology to the catalytic A subunit of Shiga toxin, protein #4 had sequence homology to anthrax lethal factor, and protein #5 had sequence homology to the protective antigen of anthrax toxin. In your expert opinion as a microbiologist and researcher in the field of bacterial pathogenesis, is there plausible cause for the DHS to be concerned that this incidence could be a potential act of bioterrorism? Be sure to state your rationale.

4. As part of a routine surveillance of several reservoirs supplying drinking water to a major metropolitan area in Washington, D.C., researchers at the Environmental Protection Agency (EPA) have identified four new *V. cholerae* strains associated with severe diarrheal disease outbreaks in the area. Being interested in understanding the pathogenesis of these new strains, the CDC researchers subjected each of these strains to a series of experiments to determine whether they contained virulence factors involved in adhesion, invasion, cell-to-cell spread, biofilm formation, or cholera toxin production. The results are shown in Table 1.
   a. Based on these results, the researchers ask you to predict the possible virulence factor(s) present in strains 1–4. Be sure to state your rationale.
   b. In your expert opinion as a microbiologist and researcher in the field of bacterial pathogenesis, is there plausible cause for the DHS to be concerned that these incidences could be potential acts of bioterrorism? Be sure to state your rationale.

Table 1.

STRAIN NO.	GENTAMICIN ASSAY (NO. OF COLONIES)			PLAQUE ASSAY (PHENOTYPE OBSERVED)	AGGLUTINATION OF RED BLOOD CELLS (PHENOTYPE OBSERVED)		RABBIT ILEAL-LOOP ASSAY DISTENSION 24 HOURS AFTER INJECTION WITH BACTERIA (VOLUME/LOOP LENGTH [ML/CM])
	WITHOUT GENTAMICIN	WITH GENTAMICIN	WITH MANNOSE AND GENTAMICIN		WITHOUT MANNOSE	WITH MANNOSE	
1	10^9	10^8	10^8	Large plaques	No clumping	No clumping	0.5
2	10^9	10^2	10^2	Large plaques	Clumping	No clumping	0.6
3	10^9	10^8	10^2	Small plaques	No clumping	No clumping	1.5
4	10^2	1	0	No plaques	Clumping	No clumping	1.7

Glossary

Note: Some terms included in this glossary have meanings in fields other than bacterial pathogenesis. This glossary focuses on the way the term is used in bacterial pathogenesis.

α-hemolysin (Hla) see **alpha-toxin**

α-hemolysis (also α-hemolytic) characterized by a dark greenish discoloration of the area around bacterial colonies on blood agar plates; used to identify certain bacteria that produce hydrogen peroxide to cause oxidation of hemoglobin, generating green methemoglobin

α hydroxyketones (AHKs) quorum-sensing molecules produced by gram-negative bacteria

α toxin see **alpha toxin**

A-B toxin (also AB-type toxin) a bacterial toxin in which the B part (domain or subunit) of the toxin responsible for binding to target cells is separate from the A part (domain or subunit) that mediates enzymatic activity or other toxic function; for some AB-type toxins, the B part often includes an additional domain (T domain) that is responsible for translocation across membranes

ABC transporter (also ATP-binding cassette transporter) a type of integral membrane pump that consists of multiple subunits; uses ATP hydrolysis to power the pump

Ablation (also ablate) (i) surgical or pharmacologic removal of selected body tissue, (ii) exogenous expression of a cell-killing toxin catalytic domain to selectively kill particular target cells, such cancer cells

Abscess (also pustule, boil) a localized collection of pus (purulent inflammatory tissue), caused by infection with bacteria or other microbe, walled off tissue pocket often fluid-filled with neutrophils and other lymphocytes and characterized by redness, pain, and swelling

Accession number a tracking number assigned to a nucleotide or protein sequence or other data deposited in a database

Accessory factor a periplasmic connecting protein that holds together the two pore-forming components of the outer membrane of the type 1 secretory system

Accessory gene regulator (Agr) the regulatory system controlling production of surface adhesins and exoproteins in *Staphylococcus aureus*

Accessory Sec system a specialized transport system found in some gram-positive bacteria

Acellular pertussis vaccine (aP) the pertussis toxin subunit form of the pertussis component vaccine; consists of pertussis toxoid with an additional component (usually an adhesin, but that differs depending on the manufacturer)

Acellular vaccine a vaccine consisting of purified, nontoxic proteins components or cellular material from killed cells, but not whole live cells

Acetylcholine (ACh) a neurotransmitter molecule whose release is inhibited by botulinum neurotoxins

ACh see **acetylcholine**

Acid-fast stain a stain used to identify acid-fast bacteria that have waxy, nearly impermeable cell walls, usually consisting of mycolic acid, fatty acids, waxes and other complex lipids; the staining procedure consists of carbol fuchsin as the primary dye, 1% solution of HCl in isopropanol or methanol as decolorizer, and a counter stain of methylene blue/malachite green dye

Acid tolerance response conditioning of bacteria to tolerate low pH, in which bacteria grown at pH 6 for a generation can survive at pH 3; regulated by Fur

Acquired immune response see **adaptive immune response**

Acquired immunity see **adaptive immunity**

ActA a surface protein of *Listeria monocytogenes* responsible for actin nucleation, involved in actin tail formation that enables the bacteria to propel through the host cell and spread from cell to cell

Actin a major protein component of the host cell cytoskeleton that polymerizes into microfilaments involved in cellular movements, morphological structures in cells and together with the protein myosin forms contractile filaments in muscle cells

Actin-based motility the mechanism by which bacteria interact with host components to form actin tails (also called comet tails), thus becoming able to spread from cell to cell

Actinomyces a genus of Gram-positive bacteria that produces many antibiotics

Activated macrophages macrophages that have been stimulated by PAMPs, cytokines and/or chemokines or exposure to antigens to have an increased killing capacity due to generation of reactive oxygen intermediates and other toxin compounds

Activating ligand (also activation ligand) any of several cellular ligands for activating receptors on the surface of natural killer (NK) cells; when an activating ligand binds to its receptor on NK cells, it triggers signal transduction that promotes NK cell activation to cause cytotoxicity and cytokine release

Activating receptor a surface activating receptor of natural killer (NK) cells that when bound to target cell ligands, triggers signal transduction that activates the NK cell to cause cytotoxicity and cytokine release

Active immunity immunity that develops as a result of an infection or immunization

Active immunization the stimulation of immune responses after exposure to an antigen that leads to protective humoral (antibody) or cellular immunity

Acute infection (also acute phase of infection, primary infection) (i) an infection characterized by rapid onset of disease, a relatively brief period of symptoms, and resolution within a few days, (ii) a systemic response against infection that has recently been acquired and the immune response recently initiated such that the microbe may not yet have been cleared, characterized by high blood levels of IgM

Acute inflammatory response see **acute-phase response**

Acute-phase proteins proteins synthesized during the acute phase of an immune response; examples are complement proteins and C-reactive protein

Acute phase response rapid production of a group of serum proteins in response to rapid onset of infection; a form of induced innate (nonspecific) immunity

Acute respiratory distress syndrome (ARDS) accumulation of fluid and PMNs in the lung; leads to insufficient gas exchange and damage to the lung

Acyl-HSL see ***N*-acyl homoserine lactones**

Adaptive immune response see **adaptive immunity**

Adaptive immune system see **adaptive immunity**

Adaptive immunity host defenses produced in response to invasion by specific bacteria or other infectious agents; involves antibodies, T cells, B cells, and activated macrophages

ADCC see **antibody-dependent cell-mediated cytotoxicity**

Adenylate cyclase (also adenylyl cyclase) a protein toxin produced by *Bordetella pertussis;* activated by intracellular calcium-bound calmodulin to catalyze the conversion of ATP to cyclic AMP

Adherent-invasive *E. coli* (AIEC) a pathotype of E. coli strains that are able to adhere to intestinal epithelial cells, can invade the host cells using actin polymerization and microtubule recruitment to trigger uptake, and can survive and replicate within macrophages

Adhesins microbial surface components that bind to the host cell surface receptors

Adhesion frequency the ratio of cell-associated colony-forming units (CFUs) to total CFUs at the end of an experiment

Adjuvant (also immune potentiator, augmenter, immunostimulants) a substance added to a vaccine that enhances antigenic stimulus or boosts the host's immune response

Adjuvant systems (AS) vaccine formulations of classical adjuvants mixed with immunomodulators that are specifically adapted to the antigen and the target population so as to activate the appropriate innate and adaptive immune responses

ADME pharmacokinetic analyses of the absorption, distribution, metabolism, and excretion of different drug doses in a host

ADP-ribosylation the transfer of the ADP-ribosyl group from NAD to a host cell protein by a bacterial protein toxin

ADP-ribosyltransferase an enzyme (or toxin catalytic domain or subunit) that catalyzes ADP-ribosylation

Adsorption the binding or adhesion of molecules, microbes or other substances to a surface matrix or to a cell surface

Adverse event any undesired medical occurrence in a patient or clinical investigation subject that occurs as a result of administration of a drug or treatment with a pharmaceutical product or procedure, which may or may not necessarily have a causal relationship with the test treatment

Aerobactin a bacterial iron-chelating siderophore produced by *E. coli* that is involved in iron sequestration in iron-poor environments

Aerobic condition where oxygen is required for growth

Aerosol a fine mist that can be inhaled

Aerotaxis movement of bacteria toward higher oxygen concentrations

Aerotolerant able to survive in an aerobic environment (describes anaerobes that can tolerate the presence of oxygen)

Affinity strength of interaction between a ligand and its receptor, such as an epitope and its cognate antigen-binding site on an antibody

Afimbrial adhesins surface proteins of bacteria important for adhesion (attachment) but not organized in pilus-like structures

Agglutination clumping of cells by specific antibody

Aggregative adherence fimbriae (AAF) a type of fimbriae (pili) produced by Shiga-like toxin-producing *E. coli* strains, which mediate cell aggregation, microcolony formation, and biofilm formation in human intestines and promote Shiga-like toxin adsorption, also promotes aggregation and biofilm formation on food and other abiotic surfaces

***agr* genes** see **accessory gene regulator**

AHKs see **a-hydroxyketones**

AHL see ***N*-acyl homoserine lactones**

AI-1 see **autoinducer 1**

AI-2 see **autoinducer 2**

AIP see **autoinducing peptide**

Alarmins endogenous, constitutively expressed, chemotactic, and immune activating proteins or peptides that act as danger signals to the immune system and are released when host cells are infected and/or damaged

Alarmone a small regulatory molecule of the host cell that is synthesized in response to a stress condition and regulates transcription of certain stress response genes; see also **(p)ppGpp**

Alkaline phosphatase (PhoA) an enzyme commonly used as a reporter group

Alpha toxin (also α-toxin, α-hemolysin, alpha-hemolysin) (i) exotoxin produced by *Staphylococcus aureus* that forms pores in human cell membranes; (ii) exotoxin produced by *Clostridium perfringens* that hydrolyzes lecithin in human cell membranes

Alternative pathway part of the innate immune response in which the complement cascade is activated by components on the bacterial cell surface

Alum an aluminum salt used as an adjuvant component of a vaccine; the only vaccine adjuvant the FDA approved for use in the United States

Alveolar macrophage a resident macrophage found in the alveoli of the lung

Aminoglycosides a family of antibiotics that bind to the 30S ribosomal subunit and prevent it from binding mRNA; includes kanamycin and gentamicin

Amplicon PCR product of amplification of DNA segments

AMPs antimicrobial peptides that act by disturbing membranes

Anabolism a set of complex metabolic pathways in the cell that are involved in synthesis of complex macromolecules from simpler components together with storage of energy (often in form of ATP)

Anaerobic growth under conditions lacking oxygen; anoxic conditions

Anergy loss of reactivity to an allergen

Animal model a species of animal that develops a disease similar to that in humans infected with the same organism

Annotation (also gene annotation) (i) a note of explanation or comment added to a text or diagram; (ii) process of identifying and labeling the locations of genes and all of the coding regions in a genome and determining the function of those genes and/or their products

Anthrax the disease caused by infection with *Bacillus anthracis*, which manifests in several forms: cutaneous anthrax characterized by swelling, edema, and necrotic lesion of the skin (called an eschar); gastrointestinal anthrax characterized by bloody diarrhea, abdominal pain, vomiting, and acute inflammation of the intestinal tract; and inhalational anthrax characterized by flu-like symptoms and respiratory distress due to swelling of lymph nodes, pulmonary edema, shortness of breath, and bloody fluid accumulation in lungs; systemic septic and toxic shock due to bacteremia, toxemia, and death

Anthrax toxin (AnTx) a multisubunit protein toxin produced and secreted by *Bacillus anthracis;* comprised of a complex between up to three subunits of one or both of the catalytic subunits, lethal factor (LF) and edema factor (EF), and a heptameric complex of the binding subunit, protective antigen (PA)

Anti-anti-sigma factors an antagonist to an anti-sigma factor, which relieves inhibition of the sigma factor activity by the anti-sigma factor

Antibacterials any agent that kills bacteria or interferes with the growth and/or reproduction of bacteria

Antibiotic a low-molecular-weight compound that can kill or inhibit growth and/or reproduction of bacteria; can be administered inside or outside the body

Antibiotic resistance the ability of bacteria to grow in the presence or to tolerate the presence of an antibiotic that would normally inhibit their growth or kill them

Antibiotic-resistant bacteria bacteria that are not killed or inhibited by antibiotics

Antibiotic tolerance the ability of bacteria to tolerate and survive antibiotic treatment even though they may be unable to divide; removal of the antibiotic restores bacterial growth

Antibody an immunoglobulin molecule produced by B cells that binds to an antigen and can mediate its neutralization and/or clearance through opsonization

Antibody-dependent cell-mediated cytotoxicity (ADCC) infected host cells that are coated with IgG and then killed by phagocytes or natural killer cells, which bombard them with toxic compounds

Antifungals (also antimycotics, fungicides, fungistatics) any agent that kills fungi or interferes with the growth and/or replication of fungi

Antigen a substance that can interact with a specific antibody (see also **immunogen, epitope**)

Antigen-binding region the portion of the Fab region of an antibody that binds to a specific antigenic determinant (epitope)

Antigen-binding sites see **antigen-binding region**

Antigen delivery system vaccine formulation systems that optimize the presentation of antigens to the immune system

Antigenic determinant a site on an antigen that binds to the antigen-binding site of an antibody; an epitope

Antigenic variation the ability of some bacteria to change the amino acid composition of their adhesins or other surface proteins to avoid the host immune system

Antigen-presenting cell (APC) an immune cell, such as a macrophage or dendritic cell, that engulfs a microbe or its products, degrades the proteins, and presents the resulting peptides on its surface to the immune system

Anti-inflammatory cytokines peptides or proteins that regulate the immune response by inhibiting the production of proinflammatory cytokines

Antimicrobial peptides a diverse class of peptides produced by the host that kill bacteria in various ways, including forming channels or holes in bacterial membranes, interfering with bacterial metabolism, or targeting cytoplasmic components; often cationic, but some are anionic

Antiparasitics any agent that kills or interferes with the growth and/or replication of parasites or parasitic organisms

Antiporter a type of efflux pump that uses uptake of protons or cations as the source of energy to pump antibiotics out of the cytoplasm

Antisense sRNAs regulatory small RNAs that have sequences complementary to certain mRNA transcripts; binding to their complementary sequences on mRNA transcripts leads to transcription attenuation or termination, translation inhibition, and enhancement or inhibition of mRNA degradation

Antiseptic an antimicrobial compound that can be applied to surfaces, such as the skin and abiotic surfaces, but cannot be ingested or injected

Antiserum serum containing antibodies against a specific antigen

Anti-sigma factors protein factors that counteract the functions of specific sigma factors by sequestering them away from RNA polymerase

Antitermination factors proteins that allow RNA polymerase to read through terminators located at the ends of genes or that bind to RNA polymerase to increase its processivity

Antitoxin (i) an antibody specific for a toxin that neutralizes its activity; (ii) a member of a toxin-antitoxin complex of a bacterial regulatory system that blocks the toxin's action

Antivirals any agent that kills a virus or interferes with its ability to infect, replicate within or be released from host cells

aP see **acellular pertussis vaccine**

APC see **antigen-presenting cell**

Apical surface the top side of an epithelial cell facing the outside of the body; portion of a cell exposed to the lumen

Apoptosis programmed cell death; occurs in normal body cells; characterized by condensation of chromatin at the boundary of the nucleus; stimulated by some bacteria and toxins

Apoptotic bodies a cellular vesicle containing cellular debris that forms during the apoptotic process

ARDB (Antibiotic Resistance Genes Database) a public database with a collection of antibiotic resistance genes that serves as information resource for the research community

ARDS see **acute respiratory distress syndrome**

Arthropod vectors a major group of host insect vectors, including mosquitos, flies, lice, fleas, ticks and mites, that serve as pathogen reservoirs and transmitters to humans and animals

AS01 see **AS04**

AS02 see **AS04**

AS03 see **AS04**

AS04 an adjuvant formulation containing monophosphoryl lipid A and alum; approved for use in humans

Aseptic free of microorganisms

Aspiration process in which fluids are introduced into or removed from body cavities

Asymptomatic carrier a person colonized by disease-causing bacteria but who does not have disease symptoms

Atg 5 see **autophagy-related protein**

Atg 8 see **autophagy-related protein**

Atg 12 see **autophagy-related protein**

Atg 16 see **autophagy-related protein**

ATP-binding cassette (ABC) transporter a large family of integral membrane-spanning proteins that utilize ATP binding and hydrolysis to transport various substances across cellular membranes, including type 1 secretion system (T1SS), drug, antibiotic, siderophore, toxin and other exoprotein efflux pumps, iron-bound siderophore or iron-binding proteins importers, sugar importers

***att* site** see **attachment site**

Attaching and effacing (also attachment and effacement) a distortion of microvilli on the apical side of an epithelial cell due to extensive rearrangement of host cell actin by EPEC strains

Attachment site the site on DNA integrons at which integrase integrates circular DNA segments; the insertion site on DNA at which phage attach and integrate into the chromosome (Campbell integration)

Attenuation (i) genetic regulation that involves RNA secondary structure; (ii) decrease in virulence of microorganisms, often through mutation, used in a vaccine

Augmenters compounds used in combination with current antibiotics to increase their efficacy or stability, thereby enhancing antimicrobial activity

Augmentin an oral antibiotic combination drug formulation consisting of amoxicillin and the β-lactamase inhibitor, clavulanic acid that is used to treat some infections with drug-resistant bacteria

Autoclaving a sterilization method that uses high-pressure steam to kill microbes

Autoimmune disease a disease that occurs when the immune system recognizes a host molecule as foreign; often induced by aberrant immune response to microbial infection

Autoimmunity (also autoimmune reactions) disease resulting from an aberrant inflammatory immune response (reaction) against an organism's own healthy cells and tissues; an immune attack against self-antigens

Autoinducer 1 (AI-1) *N*-3-oxohexanoyl-L-homoserine lactone; quorum-sensing signal molecule produced by gram-negative bacteria

Autoinducer 2 (AI-2) a small furanosyl borate diester compound used for interspecies cell-cell communication

Autoinducers small diffusible signaling molecules used by a group of bacteria to coordinate their activities

Autoinducing peptides (AIPs) small quorum-sensing signaling peptides (often containing cyclic thiolactones) used for intraspecies communication in gram-positive bacteria

Autolysins enzymes, produced by bacteria, that digest peptidoglycan and can cause lysis of bacteria; used by phage to induce bacterial cell lysis

Autophagolysosome a double-membrane vacuole that forms when an autophagosome fuses with a lysosome during autophagy

Autophagosome a double-membrane vacuole that forms when an LC3-decorated phagophore encloses cellular components targeted for degradation and/or recycling during autophagy

Autophagy a process by which components of mammalian cytoplasm or organelles are surrounded by endoplasmic reticulum and slated for destruction or recycling; similar autophagic process occurs with invading microbes, which become enclosed in an autophagophore

Autophagy-related proteins (also Atg proteins) a group of proteins that are involved in the autophagy process

Autotransporter a protein exported via a type 5 secretion system; delivered to the periplasm by the general secretory pathway and then transports itself across the outer membrane

Avidity a combination of affinity and valence; a measure of the strength of binding of antigen to antibody

Azithromycin a member of the macrolide family of antibiotics

B cell see **B lymphocyte**

B lymphocyte (B cell) the cell type that produces antibodies; see **plasma cell**

Ba a component that results from cleavage of factor B of the complement system

Bacillus (i) a rod-shaped bacterium; (ii) a genus of Gram-positive, rod-shaped bacteria belonging to the phylum Firmicutes

Bacillus anthracis a toxin-producing, spore-forming, gram-positive bacterium responsible for the disease anthrax

Bacitracin an antibiotic that interferes with the recycling of bactoprenol (lipid 55) during peptidoglycan synthesis; used topically in creams because it is too toxic for internal use

Bacteremia bacteria present in the bloodstream

Bacteria single-cell microbes lacking nuclei that, along with archaea, were the first forms of life on Earth

Bacterial interference the antagonism between bacterial species during the process of surface colonization, acquisition of nutrients, and/or establishment of infection in a host

Bacterial luciferase (Lux) see **luciferase** and **bioluminescence**

Bacterial vaginosis a disease that is characterized by a shift of the populations in the vaginal microbiota

Bactericidal a substance that kills bacteria

Bacteriocin a pore-forming toxin produced by one species of bacteria that targets another species or subspecies of bacteria

Bacteriophage a virus that infects bacteria

Bacteriostatic a substance that inhibits the growth of bacteria but does not kill them

BALT see **bronchial-associated lymphoid tissue**

Bands immature forms of PMNs

Barcode a DNA sequence introduced by PCR or transposition that is used to mark and track a DNA segment or bacterium

Basal body a complex protein structure or organelle that forms at the base of a flagellum or cilium

Basal lamina a layer of extracellular matrix to which epithelial cells attach

Basal surface the portion of a mucosal cell that is in contact with the extracellular matrix

Basement membrane a thin sheet of fibers that underlies the epithelial cell layer lining the cavities and surfaces of organs and skin or the endothelial cell layer lining the interior surfaces of blood vessels

Base-pairing sRNA regulatory small RNA (sRNA) that is able to modulate gene expression by base pairing with complementary mRNA to block translation or by unmasking or blocking the ribosome-binding site (Shine-Dalgarno site)

Basolateral membrane or surface the side and bottom portions of polarized epithelial cell membranes in a confluent monolayer; portions in contact with the extracellular matrix

Basophil a granulocyte that makes up a portion of the circulating white blood cells; contains toxic granules that are released when IgE molecules bound to the surface are cross-linked

Bb a component that results from cleavage of factor B of the complement system; complexes with activated C3 and produces C3b; C3b binds Bb, a C3 convertase

BBB see **blood-brain barrier**

B cell epitopes an antigenic determinant that specifies a part of the antigen recognized by antibodies and B cells; groups of amino acids that are located near each other on a protein's surface that can determine antigenicity through binding to the immunoglobulin or antibody

B cell receptor (BCR) a receptor on the surface of B cells that, when bound to antigens, sends activation signals to the B cell to proliferate and produce antibodies

BCR see **B cell receptor**

Bedaquiline a bactericidal drug used to treat multi-drug resistant tuberculosis; acts on the proton pump for the mycobacterial enzyme ATP synthase

Belmont Report a report issued in 1978 by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research that summarizes the ethical principles and guidelines for research involving human subjects; 3 core principles were identified as respect for persons, beneficence, and justice, and 3 primary areas of application were stated as informed consent, assessment of risks and benefits, and selection of subjects.

β-Galactosidase (LacZ) an enzyme commonly used as a reporter group using chromogenic substrates; catalyzes the hydrolysis of β-galactosides into monosaccharides

β-Glucuronidase (GUS) an enzyme commonly used as a reporter group using chromogenic substrates; catalyzes the hydrolysis of β-glucuronic acid from the nonreducing ends of glycosaminoglycans

β-Hemolysis (also β-Hemolytic) characterized by complete lysis of red blood cells on blood agar plates; the area around bacterial colonies on blood agar plates is clear (or light yellow), which is used to identify certain bacteria that produce membrane-damaging toxins that destroy the red blood cells

β-Integrins see **integrins**

β-Lactam antibiotics antibiotics that contain a β-lactam ring and act by inhibiting peptidoglycan synthesis; includes penicillins, cephalosporins, carbapenems, and monobactams

β-Lactamase an enzyme that cleaves the β-lactam ring of β-lactam antibiotics and thus inactivates them

β-Phage temperate bacteriophage that carries the gene encoding diphtheria toxin

β-Toxin a membrane pore-forming exotoxin produced by *Clostridium perfringens* that forms cation-selective pores in cell membranes

Bfp see **bundle-forming pili**

BFT (Bacteroides fragilis toxin) a zinc-dependent metalloprotease toxin produced by enterotoxigenic *Bacteroides fragilis* (ETBF) that targets cleaves E-cadherin and triggers pro-carcinogenic, multistep inflammatory cascade in colonic epithelial cells that results in colitis

Biased random walk undirected (random) motility of cells that is biased along a chemical or other concentration gradient (chemotaxis), involves sensing the environment and flagellar movement through a series of straight runs punctuated by brief periods of tumbles resulting in random changes in direction of movement

Bile salts steroids with detergent-like properties that are produced by the liver and released into the intestine; they disrupt bacterial membranes

Binary toxin (CDT) a heterodimeric subunit toxin produced by *Clostridium difficile* that catalyzes the ADP-ribosylation of host cell actin; may potentiate action of TcdA and TcdB toxins

Bioavailability measurement of the rate or fraction of an administered dose of a drug that enters the circulation and reaches the target site in a mammalian host unchanged; considered to be one of the pharmacokinetic properties of drugs

Biofilm multilayered bacterial populations embedded in a polysaccharide, protein, or DNA matrix that is attached to some surface (plastic, mucosal membrane, teeth, or implanted medical device)

Bioinformatics a field that involves design, development, management, and utilization of life science databases

Biological and Toxins Weapons Convention (BTWC or BWC) a convention held in 1975 for the purpose of ratifying the first international disarmament treaty banning the development, production, and stockpiling of biological and toxin weapons

Bioluminescence (also luciferase reaction) the production and emission of light by a living organism, such as firefly (*Photinus pyralis*), sea pansy (*Renilla reniformis*), or bioluminescent bacteria (*Vibrio fischeri*) in light organelles of marine vertebrates and invertebrates; a form of chemiluminescence where light energy is released by a chemical reaction catalyzed by luciferase (encoded by the *luc* gene), involving a light-emitting pigment, such as luciferin (encoded by the *luc* gene cluster), and oxygen

Biomarker a protein that serves as an indicator of a normal biological process or a pathogenic state in a mammalian host

Biophotonic imaging a technique for following the course of an infection in a mammalian host by monitoring the expression of the luciferase operon that has been incorporated into the chromosome of the bacterial strain being tested

Biosecurity a set of preventive measures designed to reduce the risk of transmission of infectious diseases, quarantined pests, invasive alien species, or living modified organisms

Bioterrorism the deliberate use of microbes or toxins to disrupt normal societal functions and to injure or kill people or livestock

Biothreat the threat posed by a harmful biological agent, including bacteria, fungi, viral and parasitic pathogens, and toxins produced by a variety of organisms

Bistable switches regulatory elements of complex systems that trigger gene expression and cellular functions in response to environmental signals; usually generated by a positive feedback loop with a highly sensitive regulatory step

BlaZ β-lactamase produced by *Staphylococcus aureus* in response to β-lactam antibiotics

Blood-brain barrier (BBB) the membrane (meninges) covering of the brain and spinal cord that prevents substances in blood from entering

Blood clotting (also coagulation system) the process by which blood coagulates (thickens to form a gel) with blood cells clumping to form a clot; coagulation involves activation of clotting factors, adhesion and aggregation of blood platelets, and deposition and maturation of crosslinked fibrin to plug the site of injury and stop the bleeding

Boiling heating of water to 100°C (212°F); brining a liquid to the temperature at which it bubbles and turns to vapor

BoNT see **botulinum neurotoxin**

***Bordetella* tracheal cytotoxin (TCT)** a peptidoglycan fragment of *Bordetella pertussis* membrane that kills ciliated cells, stimulates the release of IL-1, and triggers the coughing response in the disease whooping cough

Borrelia burgdorferi a tick-borne spirochete bacterial species that causes Lyme disease

Botulism paralysis resulting from intoxication with botulinum neurotoxin

Botulinum neurotoxin (BoNT) a protein neurotoxin produced by *Clostridium botulinum* that enters motoneurons and catalyzes the zinc-dependent proteolytic cleavage of specific SNARE proteins involved in the release of the neurotransmitter acetylcholine, resulting in flaccid paralysis; there are at least seven distinct serotypes (A, B, C, D, E, F, and G); also related to tetanus toxin (TeNT) from *Clostridium tetani*

Bradykinin a vasoactive peptide of 9 amino acids that enhances extravasation and mediates a proinflammatory response due to increased vasodilation and contraction of smooth muscle

Broad spectrum of activity description of an antibiotic that is effective against many different types of bacteria

Bronchial-associated lymphoid tissue (BALT) the respiratory tract equivalent of the gastrointestinal-associated lymphoid tissue

Bt toxin (also δ-endotoxin, crystal protein, or Cry protein) a diverse family of insecticidal protein toxins produced by the *Bacillus thuringiensis*, a soil-dwelling bacterium that is a gut pathogen of many insect species; usually encoded by *cry* genes located on a plasmid, Cry toxins insert into the insect gut membrane and form a pore, eventually killing the insect

BTWC see **Biological and Toxins Weapons Convention**

Buboes (also singular bubo) enlarged, swollen, painful and highly inflamed lymph nodes in the armpit, neck, thighs or groin, symptomatic of bubonic plague disease where the *Yersinia pestis* bacteria replicate in the lymph nodes

Bubonic plague one of three disease manifestations of infection with *Yersinia pestis*; after being bitten by an infected flea, the bacteria localize to lymph nodes and cause buboes and then spread systemically (bacteremia)

Bullous impetigo (also scalded-skin syndrome) a type of skin infection characterized by painless blister-like lesions; caused by toxins produced by *Staphylococcus aureus*

Bundle-forming pili (Bfp) the type IV pili of EPEC thought to be important in self-aggregation of bacteria (microcolony formation)

Buruli ulcer a skin and soft tissue lesion caused by *Mycobacterium ulcerans*, characterized by chronic, debilitating necrotizing tissue damage due to production of an immunosuppressing mycolactone toxin that dampens the immune system and acts as a necrotizing factor that results in cell and tissue death

BWC (or BTWC) see **Biological and Toxins Weapons Convention**

C1 a complement component important in the classical pathway; activated by bacterium-antibody complexes

C1a a complement component that cross-links Fc regions of IgG or IgM; part of the classical pathway; cleaves C4 and C2

C2 a complement component activated by C1 in the classical pathway

C2a a complement component resulting from cleavage of C2; binds C4b to form C3 convertase

C2b a complement component resulting from cleavage of C2

C3 a complement component that is cleaved into C3a and C3b by C3 convertase

C3 convertase a complex of complement components, comprised of either C3bBb or C2aC4b, that convert C3 to C3a and C3b

C3a a complement component that results from the proteolytic cleavage of C3; acts as a vasodilator

C3b a complement component that results from the proteolytic cleavage of C3; binds to bacterial surfaces and potentiates their opsonization; forms part of C3 convertase

C3b/C4b receptor (also called **complement receptor 1, CD35)** a membrane glycoprotein on the surface of phagocytic antigen-presenting immune cells such as dendritic cells (DCs) and macrophages that mediates opsonization, processing and clearance of microbes coated with C3b and/or C4b; can also act as a negative regulator of the complement cascade through the classical and alternative pathways

C4 a complement component that is cleaved by activated C1 of the classical pathway or components of the MBL pathway

C4a a complement component that results from cleavage of C4; acts as an anaphylatoxin to mediate local inflammation at the site of infection

C4b a complement component that results from cleavage of C4; binds to C2a to form C3 convertase

C4b-binding protein (C4BP) a large glycoprotein that binds to C4b (and C3b) to accelerate the decay of C3-convertase; inhibits the classical and lectin complement pathways; acts as a cofactor for serine protease factor I which cleaves C4b (and C3b)

C5 a complement component that is cleaved to C5a and C5b by C5 convertase

C5 convertase a complement component comprised of a complex between C3 convertase and C3b that converts C5 to C5a plus C5b

C5a a complement component that results from proteolytic cleavage of C5; acts as a chemoattractant for PMNs

C5b a complement component that results from proteolytic cleavage of C5; C5b recruits C6, C7, C8, and C9 to form the membrane attack complex (MAC) that pokes holes in membranes of gram-negative bacteria

Calcium-calmodulin-dependent adenylate cyclase (Cya) a protein toxin produced by *Bordetella pertussis* that converts ATP into cyclic AMP; only active inside host cells, since it depends on calcium-calmodulin for activity

Calcium mobilization release of calcium from intracellular stores triggered by interaction of a cell with bacteria or bacterial toxins

Calmodulin a mammalian protein that binds calcium; required to activate *Bordetella* adenylate cyclase

Calprotectin a cation-binding protein complex of S100A8 and S100A9 that in the presence of calcium chelates and sequesters manganese and zinc ions (can also bind iron); stored in neutrophil granulocytes and released during inflammation; has antimicrobial properties through metal sequestration

Capsule an extracellular network, often of exopolysaccharides, but sometimes peptides, that covers the cell surfaces of some bacteria and usually interferes with phagocytosis

Capture or sandwich ELISA see **enzyme-linked immunosorbent assay (ELISA)**

Carbapenem a class of β-lactam antibiotics

CARD (Comprehensive Antibiotic Resistance Database) a rigorously curated collection of characterized genes that confer antibiotic resistance determinants, includes the antibiotics and their targets along with antibiotic resistance genes, associated proteins and literature references

Cardiotoxin a toxin that targets heart cells

Caries (also dental caries, cavities) the decay of a tooth or bone caused by the action of bacteria and the ensuing inflammatory response

Carrier (also asymptomatic carrier) an apparently healthy person who displays no signs or symptoms of disease, but who harbors pathogenic microbes and can transmit them to others

Carrier state the condition of harboring an infectious organism without manifesting any symptoms of disease

CARVER+Shock (criticality, accessibility, recuperability, vulnerability, effect, recognizability, and shock effect) an assessment method for identifying the most vulnerable target sites for introduction of contamination within the food-processing system

Caspase-1 (also IL-1 converting enzyme, ICE) an enzyme that proteolytically cleaves precursors of the inflammatory cytokines IL-1β and IL-18 into active mature peptides

CAT see **chloramphenicol acetyltransferase**

***cat* gene** a gene that encodes chloramphenicol acetyltransferase (CAT), which imparts resistance to chloramphenicol and is used as a reporter for transcriptional regulation

Catabolism the set of metabolic pathways involved in breakdown of large macromolecules (such as proteins, DNA, RNA, lipids, and polysaccharides) into smaller molecules (such as amino acids, nucleotides, fatty acids, and monosaccharides) that are either used to yield energy or recycled for use in anabolic reactions to build new macromolecules.

Catabolite control protein E (CcpE) a regulatory protein of Staphylococcus that mediates carbon catabolite repression and the expression of virulence factors such as exotoxins and capsule, and proteins involved in biofilm formation

Catechol a class of siderophore

Cathelicidin an antimicrobial peptide produced by host immune cells

CD1 (also CD1 molecules) a protein molecule on the surfaces of antigen-presenting cells that presents lipid or glycolipid antigens to the immune system

CD4 (also CD4 molecules) a transmembrane glycoprotein receptor expressed on the surfaces of helper T cells, regulatory T cells, monocytes, macrophages, and dendritic cells that acts as a coreceptor to assist the T cell receptor (TCR) to activate its cognate T cell following interaction with antigen-bound MHC II complex on an antigen-presenting cell

CD4$^+$ T cells see **T helper (Th) cells**

CD8 (also CD8 molecules) a transmembrane glycoprotein receptor expressed on cytotoxic T cells, natural killer cells, and dendritic cells that acts as a coreceptor to assist the T cell receptor (TCR) to activate its cognate T cell following interaction with antigen-bound MHC I complex on a cell

CD8 T cells see **cytotoxic T cells**

CD14 a pattern recognition receptor on monocytes and macrophages that binds LPS–LPS-binding protein complex and acts as a coreceptor (along with TLR-4 and MD-2) for detection of bacterial LPS by the innate immune system

CD35 (complement receptor 1, CR1) see **C3b/C4b receptor**

CD46 (cluster of differentiation 46, membrane cofactor protein) an inhibitory complement receptor that mediates cleavage and inactivation of complement components C3b and C4b

CD55 see **decay-accelerating factor**

CD59 (MAC-inhibitory protein, MAC-IP) a membrane-bound glycoprotein that can prevent complement component C9 from polymerizing and forming the membrane attack complex

Cdc42 a small G protein (GTPase) involved in regulation of the mammalian cell cycle that is targeted by toxins A and B of *Clostridium difficile*

cDNA complementary DNA biochemically made from RNA by reverse transcription using a reverse transcriptase enzyme

CDC see **Centers for Disease Control and Prevention**

CDT see **binary toxin**

CdtA one of the subunits of the ternary complex of cytolethal distending toxin (CLDT or CDT) from *Escherichia coli, Campylobacter,* and several other gram-negative pathogens that, along with CdtC, mediates the receptor binding and translocation of the catalytic subunit, CdtB

CdtB one of the subunits of the ternary complex of cytolethal distending toxin (CLDT or CDT) from *Escherichia coli, Campylobacter,* and several other gram-negative pathogens that mediates host cell DNA damage to cause cell cycle arrest in G_2 phase

CdtC one of the subunits of the ternary complex of cytolethal distending toxin (CLDT or CDT) from *Escherichia coli, Campylobacter,* and several other gram-negative pathogens that, along with CdtA, mediates the receptor binding and translocation of the catalytic subunit, CdtB

CdtR a positive regulator of the CDT toxin genes

Cell-mediated immunity (CMI) adaptive immunity due to activation of antigen-specific cytotoxic T cells, macrophages, and natural killer cells; does not involve antibodies or complement

Cell-to-cell spread a situation where bacteria growing inside one host cell burst out and spread to adjacent host cells; may lead to production of a plaque; characteristic of actin-based motility (via actin tail formation)

Cell wall hydrolases a group of enzymes that cleave covalent bonds within cell wall glycan strands or the peptide side chains and are responsible for cell wall remodeling to incorporate new peptidoglycan and to accommodate changes in cell shape

Centers for Disease Control and Prevention (CDC) a national government agency whose mission is to provide the expertise, information, and tools that people and communities need to protect their health through health promotion, prevent of disease, injury and disability, and preparedness for new health threats

Cephalosporins a class of β-lactam antibiotics

CF see **cystic fibrosis**

CFTR see **cystic fibrosis transmembrane conductance regulator**

CFU see **colony-forming unit**

Chaperone a protein that aids in the folding of proteins after translation or of RNA molecules after transcription; a protein that binds and stabilizes another protein or brings another protein to a particular site in the cell; a protein that potentiates pairing between small RNAs and mRNAs

Chaperone-usher system a complex protein system in the periplasm of gram-negative bacteria that plays a role in the assembly of extracellular pili; requires a number of auxiliary proteins

Chelocardin an antibiotic in the tetracycline family that acts by disrupting the function of the cytoplasmic membrane

Chemoattractants soluble proteins, peptides (such as immunomodulatory chemokines), or small organic or inorganic molecules that can induce chemotaxis (movement toward a chemical gradient) in motile cells

Chemokines small glycopeptides (8 to 10 kDa) produced by many human cell types; they organize activities of cells of innate and adaptive defenses

Chemorepellents soluble proteins, peptides (such as immunomodulatory chemokines), or small organic or inorganic molecules that can induce chemotaxis (movement away from a chemical gradient) in motile cells

Chemotaxis the attraction and movement of bacteria toward or away from a particular chemical substance (along a chemical gradient)

Chemotherapy treatment of disease with drugs

Chloramphenicol acetyltransferase (CAT) an enzyme that inactivates chloramphenicol and imparts antibiotic resistance; often used as a reporter for gene transcription

Chloroplasts a plant and algal organelle that conducts photosynthesis through capture of sunlight by the pigment chlorophyll and conversion with water into ATP and NADPH and oxygen; chloroplasts replicate their own DNA and are thought to derive from an ancestral photosynthetic cyanobacterium that was acquired by an early eukaryotic cell

Cholera toxin (CT) a multisubunit AB_5-type exotoxin complex produced by *Vibrio cholerae* that ADP-ribosylates and constitutively activates the heterotrimeric Gs protein involved in regulation of mammalian adenylate cyclase; results in elevated intracellular cyclic AMP levels

Choline-binding proteins (CBPs) a family of surface proteins noncovalently bound to the phosphorylcholine moiety of the cell well of *Streptococcus* by a conserved choline-binding domain

Chromogenic substrate a substrate that changes color on plates or in liquid medium if a particular enzyme is present (e.g., X-Gal)

CI see **competitive index**

Cilia surface structures of eukaryotic epithelial cells that move mucus over surfaces

Ciliary dyskinesia a genetic disorder that causes defects in the action of cilia lining the respiratory tract, fallopian tube, and sperm cell flagellum

Ciliated columnar cells mucosal cells that have cilia on their apical surfaces; commonly found lining the respiratory tract and fallopian tubes

Cipro see **ciprofloxacin**

Ciprofloxacin a member of the fluoroquinolone antibiotic family

Class I major histocompatibility complex (MHC I) a major histocompatibility type that when complexed with an epitope triggers activation and proliferation of cytotoxic T cells and natural killer cells; found on most cells in the body

Class II major histocompatibility complex (MHC II) a major histocompatibility type that when complexed with an epitope leads to activation and proliferation of helper T cells; found only on a few immune cell types (e.g., B cells and APCs)

Classical complement pathway a complement pathway that is activated by antigen-antibody complexes

Classical strain the O1 strain of *Vibrio cholerae* first associated with pandemics

Clavulanic acid a suicide substrate inhibitor of serine-type β-lactamases that is used in combination therapy with β-lactam antibiotics to enhance their stability by preventing their degradation

CLDT see **cytolethal distending toxin**

Clindamycin a member of the lincosamide antibiotic family

Clinical & Laboratory Standards Institute (CLSI) a not-for-profit membership organization dedicated to fostering excellence in laboratory medicine by providing guidelines and standard for laboratory practices

Clumping factor (ClfA) a virulence factor of *Staphylococcus aureus* that binds to fibrinogen that causes clumping of blood plasma when the bacteria at added to human plasma; also binds complement regulatory factor I to increase factor I cleavage of C3b

CMI see **cell-mediated immunity**

CMT see **cytolysin-mediated translocation**

CNFs see **cytotoxic necrotizing factors**

CNS see **coagulase-negative staphylococci**

Coagulase a bacterial enzyme that induces coagulation of blood or plasma by cleaving fibrinogen to fibrin

Coagulase-negative staphylococci strains of staphylococci, such as *Staphylococcus epidermidis* and *Staphylococcus saprophyticus,* that do not produce coagulase

Coagulase-positive staphylococci strains of staphylococci, such as *Staphylococcus aureus,* that do produce coagulase

Coccus (plural cocci) a sphere-shaped bacterium

Cohort as group of subjects in a clinical trial who share one or more common traits or defining characteristics, life history, similar experiences or illness, genetic makeup or background; a group observed over a period of time under certain conditions during a clinical trial

Cold pasteurization see **food irradiation technology**

Colibactin a polyketide-derived cyclomodulin-like bacterial toxin found in members of the family *Enterobacteriaceae* (including *E. coli* and *Klebsiella pneumoniae*) that interferes with the eukaryotic cell cycle by causing double-stranded DNA breaks, chromosome aberrations, and cell cycle arrest in G2/M phase

Colicin A a channel/pore-forming transmembrane toxin that depolarizes the cytoplasmic membrane, leading to dissipation of cellular energy

Colicin Ia a type of bacteriocin made by *E. coli* that acts against other *E. coli* to kill them by forming a voltage-dependent channel in the inner membrane of the bacteria

Coliforms a broad group of rod-shaped, Gram-negative, non-spore-forming and motile/nonmotile bacteria found in the environment, including feces of humans and animals, that can ferment lactose with production of acid and gas at 35-37°C; the presence of coliform bacteria in drinking water indicates the presence of potentially pathogenic bacteria

Colitis inflammation of the colon

Collaborative cross (CC) a multiparent panel of recombinant inbred (RI) mouse strains derived from eight founder laboratory strains; serves as a common mouse reference panel for mouse disease models to overcome the limitations of existing mouse genetic resources for analysis of phenotypes caused by combinatorial allele effects

Collagenases enzymes that hydrolyze (cleave) the peptide bonds of collagen, thereby destroying extracellular structures that comprise connective tissue

Collagen the main structural protein in the extracellular space of connective tissue in the body; collagen strands wind together to form triple-helices of elongated fibrils of fibrous tissues

Collectin pathway see **mannose-binding lectin (MBL)**

Collectins a family of calcium-binding lectins that are soluble pattern recognition receptors involved in innate immune response; includes mannose-binding lectin

Colonization the ability of a bacterium to remain at a particular site in the host and multiply there; can be asymptomatic

Colonization factor antigens I and II different types of pili produced by ETEC

Colony a discrete mass of cells derived from a single bacterial cell or a chain of bacterial cells

Colony-forming unit (CFU) a measure of the number of viable bacteria in a sample; each colony on an agar plate arises from a single bacterium or a chain of bacteria

Columnar epithelium tall, thin epithelial cells

Combinatorial chemistry an approach to generate new synthetic antibiotics and drugs; a large variety of chemical groups are added to a scaffold, and the resulting array is tested for activity as a mixture

Comedo (also called a pimple, acne, zit, blackhead, whitehead) an open (blackhead) or closed (whitehead) skin pore or hair follicle clogged with oil, dead skin cells, and bacteria

Commensal microbiota see **resident microbiota**

Common Rule a rule or baseline standard of ethics in the United States regarding biomedical and behavioral research involving human subjects and protections for certain vulnerable research subjects

Competent a state in which bacteria are capable of taking up extracellular DNA from the medium and incorporating homologous sequences into their chromosomes by recombination

Competitive index (CI) [output ratio (CFU mutant/CFU wild type)]/[input ratio (CFU mutant/CFU wild type)]; a means for determining whether a mutation gives a bacterium a competitive advantage in an animal model of infection or mixed-growth experiment

Complement see **complement system**

Complement activation proteolytic cleavage of complement components to produce activated proteins that attract phagocytes, cause lysis of gram-negative bacteria, and opsonize bacteria

Complement system a group of plasma proteins that mediate innate immunity and the inflammatory response when activated

Complementation test a test to determine whether two different mutants with the same phenotype are due to mutations in the same gene; done by expressing a wild-type copy of the gene from a second location in the chromosome or from a plasmid

Complex trait analysis (CTA, also genome-wide complex trait analysis) a statistical method for genetic analysis for estimation of component variance; quantification of the total contribution that a particular subset of genetic variants or single-nucleotide polymorphisms (SNPs) make to the heritability of a trait; estimates the genetic relationship between two individuals based on their genome-wide SNPs

Concatenate to link multiple things together in a chain or series, such as to link multiple genes in tandem

Conduction heating (also thermal conduction, conduction heat transfer) the transfer of heat by means of molecular excitement within a solid material without bulk motion

Conjugate vaccine a vaccine comprised of a polysaccharide antigen covalently linked to a carrier protein; forces the polysaccharide to be processed as a protein-like antigen through the T cell-dependent pathway

Conjugation the transfer of DNA directly from one bacterial cell to another; mediated by transfer *(tra)* genes and a specific pilus

Conjugative plasmid a plasmid that carries genes encoding proteins needed for the conjugation process

Conjugative transposons transposons that can transfer themselves from the genome of the donor to the genome of the recipient

Constant region (Fc) the portion of an antibody molecule that binds complement component C1 and receptors on phagocytes and other immune cells; see **Fc**

Contigs (from continuous) a set of overlapping DNA segments that together represent a consensus region of the DNA sequence

Continuous epitope a single linear sequence of an antigen recognized by an antibody

Coordinated regulation regulation of multiple genes in response to a particular signal; see **operon** and **regulon**

Coordinate regulation of virulence simultaneous regulation of multiple virulence genes or operons in response to a particular signal

Core genome the set of genes present in and shared by all strains of a species

Cosmetic botulism botulism (or paralysis) resulting from cosmetic application of botulinum neurotoxin

Coverage (or depth) in regard to DNA sequencing, the number of unique DNA sequence reads that include a given nucleotide position in the reconstructed DNA sequence

CpG oligonucleotides see **CpG-rich DNA**

CpG-rich DNA a sequence of DNA present in bacteria but not mammals; acts in stimulation of innate immunity by a toll-like receptor

C-reactive protein (CRP) an acute-phase protein produced by the liver that appears in blood serum in response to inflammation

CRISPR (clustered regularly interspaced short palindromic repeats) direct repeats scattered throughout the chromosomes of many bacteria; function as a quasi-immune system against exogenous genetic elements

CRISPR-Cas see **CRISPR-Cas systems**

CRISPR-Cas systems (clustered regularly interspaced short palindromic repeats) a family of repetitive DNA sequences found within the genomes of bacteria and archaea that are derived from DNA fragments from phage/plasmids that have previously infected the microbe and that play a role in protecting those bacteria or archaea from subsequent exposure to those plasmids or bacteriophage/virus; a form of bacterial immunity that prevents bacteriophage infection, plasmid conjugation and natural transformation by degrading the foreign DNA that enters the cell

CRISPR-Cas9 (see also CRISPR-Cas systems) Cas9 enzyme uses CRISPR sequences as a guide to bind and cleave specific strains of DNA that are complementary to the CRISPR sequence; along with engineered guide RNA (gRNA), constructed from the tracrRNA that binds Cas9 and complementary DNA to the target site, used to edit genes within genomes (genome editing)

Crohn's disease an inflammatory bowel disease (IBD) that is characterized by mild to severe inflammation of the digestive tract

CRP see **C-reactive protein**

crRNA (also trans-activating tracrRNA) see **CRISPR-Cas9**

Cryptic gene phenotypically silent DNA sequences that appear to be genes with potential function, but are not normally expressed unless activated through mutation, recombination, insertion elements, or other genetic alteration event

Crystal or Cry toxin (also called delta endotoxin, δ-endotoxin) a group of insecticidal toxins that produced by *Bacillus thuringiensis* as crystalline inclusions during stationary phase of bacterial growth and that are cytotoxic against larvae of insects

CT see **cholera toxin**

CTLs see **cytotoxic T cells**

C-type lectin a type of lectin that requires calcium for binding; involved in recognition of cells infected by viruses

Cuboidal epithelium cube-shaped epithelial cells

Culture microorganisms growing in liquid or on solid medium

Cya see **calcium-calmodulin-dependent adenylate cyclase**

Cystic fibrosis (CF) a disease caused by a defect in chloride secretion due to a mutation in the gene for a chloride transmembrane conductance regulator protein (CFTR) that regulates production of sweat, digestive juices, and mucus; characterized by production of thick mucin in the lungs that causes difficulty in breathing

Cystic fibrosis transmembrane conductance regulator (CFTR) an ABC transporter-like chloride ion channel protein that transports chloride ions across epithelial cell membranes

Cytokine technology a technique for modulating the immune system

Cytokines signaling proteins (8 to 30 kDa) produced by some mammalian cells in response to stimuli; mediators of inflammation and septic shock

Cytolethal distending toxin (CLDT, or CDT) a heterodimeric subunit AB toxin (comprised of CdtA, CdtB, and CdtC subunits) produced by EPEC, *Campylobacter,* and several other pathogenic bacteria that causes mammalian cell cycle arrest in G_2 phase; leads to enlargement and distension of host cells

Cytolysin a toxin, usually membrane pore-forming that causes lysis of cells

Cytolysin A (ClyA, also HlyE) a membrane pore-forming toxins produced by certain pathogenic strains that lyses cells

Cytolysin-mediated translocation (CMT) a proposed mechanism by which gram-positive bacterial effector proteins are secreted by the general secretory system and delivered directly into host cells through a pore in the eukaryotic membrane created by a secreted pore-forming toxin

Cytoskeleton a complex array of proteins that gives shape to eukaryotic cells

Cytotoxic necrotizing factors (CNFs) a family of AB-type protein toxins produced by some EPEC and *Yersinia* strains that catalyze the deamidation and activation of small Rho GTPases, which causes cytoskeletal rearrangements

Cytotoxic T cells $CD8^+$ T cells (with CD8 antigen on their surfaces) that kill host cells displaying foreign antigens on their surfaces through the MHC I complex

Cytotoxic T lymphocytes (CTLs) see **cytotoxic T cells**

Cytotoxin a toxin that kills mammalian cells

D the diphtheria toxoid component of the DTP, DTaP or DaPT vaccine

DAF see **decay-accelerating factor**

Dalfopristin a streptogramin antibiotic

Daptomycin a glycopeptide antibiotic; commercially called Cubicin

Databases centralized repositories for sequencing, transcriptome, and proteomic information; most are web based and freely available online (e.g., NCBI)

DCs see **dendritic cells**

DDBJ DNA Databank of Japan; Japan's nucleotide database

Debilitation loss of health

Debridement the surgical removal of dead tissue

Decay-accelerating factor (DAF, also CD55) a membrane glycoprotein that regulates the complement system on the mammalian cell surface by preventing assembly of or accelerating the disassembly of the C3bBb complex and blocking formation of the membrane attack complex; the site where *Helicobacter pylori* binds to gastric epithelium

Deconvolution determination of which compound in a large group is the one with the desired activity; used in combinatorial chemistry screening for antibiotics or inhibitors

Deep sequencing in regard to DNA sequencing, the general concept of aiming for a high number of unique sequence reads within each region of sequence

Defensins a specific type of antimicrobial peptide comprised of cysteine-rich cationic peptides and used by mammalian host cells to kill bacteria

Degradative enzymes lysosomal proteins, e.g., proteases and lysozyme, that destroy bacterial surface components

Delta-toxin a membrane pore-forming exotoxin (hemolysin) produced by *Clostridium perfringens*

Dendritic cells (DCs) antigen-presenting cells (APCs) that process invading bacteria and activate host immune defenses

Dental plaque see **plaque**

Deoxyribonuclease an enzyme that degrades DNA

Derivative toxin the toxic portion of botulinum toxin (see **progenitor toxin**)

Dermis the connective tissue below the epidermis of the skin

Desmoglein (Dsg) a family of cadherin proteins on the surfaces of epithelial cells that form desmosomes, which join cells to one another; maintains keratinocyte cell-cell adhesion

Desmosomes protein structures that hold epithelial cells together

Desquamation shedding of dead cells of the epidermis

Diapedesis see **transmigration**

Diarrhea abnormal frequency of bowel movements and fluidity of stool

Diarylquinoline a class of antibiotics used to treat tuberculosis that target the c subunit of mycobacterial ATP synthase and block synthesis of ATP

DIC see **disseminated intravascular coagulation**

Differential toxicity a desirable trait of an antibiotic in which it is more toxic to bacterial cells than to the human body

Diffusely adherent *E. coli* (DAEC) a group of pathogenic E. coli strains that express Afa/Dr-like adhesins that promote urinary tract infections (UTIs)

Digitonin a steroidal saponin that acts as a detergent to solubilize water-soluble membrane lipids and permeabilize host cell membranes

Diphthamide a post-translationally modified histidine found in mammalian translation elongation factor EF-2 that is ADP-ribosylated by diphtheria toxin and *Pseudomonas* exotoxin A (ExoA)

Diphtheria the deadly disease caused by infection with Corynebacterium diphtheriae and intoxication with diphtheria toxin (DT), which results in killing of cells lining the throat (forming a scab-like pseudomembrane) and upper respiratory tract, causing swelling of the lymph nodes and leading to difficulty in breathing, cardiac arrhythmias, myocarditis, inflammation of nerves, kidney problems and heart failure

Diphtheria toxin (DT) an AB protein toxin produced by *Corynebacterium diphtheriae* that binds and enters cells and blocks protein synthesis by ADP-ribosylation of mammalian elongation factor 2 (EF-2)

Diphtheria toxin repressor (DtxR) a Fur-like regulatory protein of *Corynebacterium diphtheriae* that mediates iron regulation of diphtheria toxin gene transcription

Diphthine a deamidated version of diphthamide found in *Archaea*

Diplococci bacterial cocci arranged as pairs of cells, e.g., pneumococci

Direct ELISA see **enzyme-linked immunosorbent assay**

Discontinuous epitope the nonlinear portions of an antigen recognized by an antibody that are adjacent to each other due to the structural conformation of the antigen

Disease damage to the host, such as that caused by an infection, which often manifests symptoms

Disease transmission the means by which an infectious agent spreads from one host to another

Disinfectant an antimicrobial compound applied to inanimate objects; often too toxic for internal application

Disseminated intravascular coagulation (DIC) formation of numerous small blood clots that obstruct peripheral blood vessels; a symptom of septic shock

Diversity outbred (DO) a stock resource of genetically diverse mice that may be used as a tool for genetic mapping and validation of quantitative trait loci (QTLs) linked to disease susceptibility, drug resistance or behavioral phenotypes

DNA chip the array of DNA on a microarray (usually a glass slide or membrane)

DNA database a resource containing sequences of nucleotides in specific DNA samples, especially genomes

DNA invertase a site-specific DNA recombinase that catalyzes the inversion of a DNA segment

DNA methylase an enzyme that can transfer methyl groups to DNA bases; an enzyme that catalyzes DNA methylation

DNA methylation addition of methyl ($-CH_3$) groups to DNA that changes the properties of the DNA, in promoter regions often causes repression of gene transcription

DNA microarray an orderly arrangement of DNA oligonucleotides corresponding to genes of interest

DNA sequencing technologies (Next-generation sequencing technologies) current sequencing approaches that enable rapid, high-throughput, low-cost, high sensitivity and massive data output from sequencing of single or multiple genes to genomes to complex mixtures of genomes; features automation, parallel processing and running of samples to produce millions of sequences concurrently; methods currently applied include: PacBio SMART cell sequencing with read lengths up to 100,000 bases; Illumina sequencing with millions to billions of short read lengths of 50 to 600 bases; Nanopore sequencing in real time with read lengths up to 2 billion bases

DNA spacer sequences (also intergenic spacer DNA) sequences of noncoding DNA between genes (intergenic), particularly between many tandemly repeated copies of the genes (such as between ribosomal RNA genes or CRISPR repeats)

DNA-uptake sequence (DUS) short DNA sequences scattered throughout the genomes of some naturally competent bacteria that serve as recognition sites for binding and uptake of that DNA by certain bacteria

DNA vaccine a vaccine consisting of DNA encoding a vaccine protein target that is injected into muscle cells, where the protein is synthesized and stimulates an immune response

DNase see **deoxyribonuclease**

Donor see **donor cell**

Donor cell a cell from which DNA is donated (to a recipient cell) during genetic exchange

Dormancy a state in which an organism's metabolic activity (growth, development, physical activity) is temporarily halted or greatly reduced, helping to conserve energy; the organism is alive but not actively growing; a state adopted by many bacteria under unfavorable environmental conditions (or in the presence of selective pressure such as antibiotics)

Double-blind study a clinical trial where the neither the participants nor the experimenters know which subject cohort is the control and which is receiving the treatment; research trial protocol utilized to prevent introduction of bias to the interpretation of the research results

Doxycycline a tetracycline-like antibiotic used to treat variety of bacterial infections (also parasitic infections); inhibits bacterial protein synthesis by binding to the 30S ribosomal subunit and blocking the attachment of charged aminoacyl-tRNA to the A site of the ribosome

Druggable a term used in drug discovery to describe a biological target (such as protein) that is known to bind with high affinity to a drug

Drug resistant resistant to at least one class of drug

Dsg see **desmoglein**

DT see **diphtheria toxin**

DT vaccine a divalent vaccine against diphtheria and tetanus comprised of the diphtheria toxoid and tetanus toxoid

DTP (or DTaP) vaccine a trivalent vaccine against diphtheria, tetanus, and pertussis comprised of the diphtheria toxoid, tetanus toxoid, and either dead *Bordetella pertussis* bacteria or acellular components (pertussis toxoid plus pertactin or another adhesin)

DtxR see **diphtheria toxin repressor**

Dual RNA-Seq technology transcriptomic profile of gene expression simultaneously in an infecting bacterium and its infected host

Dual-use agent biological agents that can be used in benign ways for beneficial purposes such as biomedical or biotechnological applications, but can also be used in malicious ways as bioweapons or biothreats

Dual-use research of concern (DURC) biological or chemical research that can be reasonably anticipated to provide knowledge, information, products, or technologies that could be used for nefarious purposes or could be directly misapplied to pose a significant threat with broad potential consequences to public health, safety, economic or environmental wellbeing or national security

DUS see **DNA uptake sequence**

Dysbiosis a perturbation or imbalanced composition of the normal microbiota that results in dysfunction, disease or other pathogenic outcome

Dysentery a type of diarrhea in which stools contain blood and mucus; associated with infection by Shiga toxin- or Shiga-like toxin-producing bacteria

EAE see **enterocyte attachment and effacement**

ECM see **extracellular matrix**

Edema excessive fluid in the tissues; associated with the edema factor component of anthrax toxin

Edema factor (EF) the adenylate cyclase subunit of the anthrax toxin complex that increases intracellular cAMP levels in eukaryotic cells and consequently results in tissue edema

Edible vaccines plants that have been genetically modified to produce a bacterial protein antigen for oral vaccination

EF-2 see **elongation factor 2**

Effector proteins see **exoenzymes**

Efflux pump cytoplasmic membrane proteins that mediate resistance to tetracycline, macrolides, quinolones, and other classes of antibiotics by pumping them out of the bacterial cytoplasm

EHEC see **enterohemorrhagic *Escherichia coli***

Ehrlichia chaffeensis an arthropod-borne pathogen that causes human monocytotropic ehrlichiosis (HME) disease

EIEC see **enteroinvasive *Escherichia coli***

El Tor the O1 strain of *Vibrio cholerae* associated with more recent pandemics

Elastase an enzyme that degrades the elastin component of the extracellular matrix; may be important in causing lung damage in *Pseudomonas aeruginosa* infections

Electron beams streams of high-energy electrons produced by an electron gun and used to irradiate food

Elimination refers to the complete and permanent worldwide reduction to zero of new cases of the disease through deliberate efforts such as immunization

ELISA see **enzyme-linked immunosorbent assay**

Elongation factor 2 (EF-2) a protein that plays an essential role in host cell protein synthesis; target of diphtheria toxin and *Pseudomonas* ExoA toxin

EMBL NDB European Molecular Biology Laboratory Nucleotide Database; Europe's library of nucleotide sequence data

Emerging infectious diseases new diseases that appear or become known, often due to increased human contact with a microbe that has been around for years

Endemic continually present at low levels in the community (describing a disease)

Endocarditis inflammation of heart valves

Endocytosis engulfment (usually receptor mediated) of extracellular material into an endosome or vacuole by a eukaryotic cell; bacteria and bacterial toxins often trigger their uptake into cells via this mechanism

Endoflagella the specialized flagella located in the periplasm of spirochetes that are tightly wound around the cork-screw shape that together to form an axial filament

Endospore a survival form of some bacteria that protects against environmental extremes; a dormant, hardy, non-replicating form of bacteria that are resistant to most agents that kill vegetative bacteria

Endosymbiont a microbe that lives inside the cells of its symbiotic partner

Endothelial cells cells constituting the endothelium

Endothelium a thin layer of cells lining the interior surfaces of blood vessels, lymphatic vessels, and the heart

Endotoxic shock see **septic shock**

Endotoxin see **lipopolysaccharide**

***ent* genes** genes encoding the enterotoxins of *Staphylococcus aureus*

Enteric relating to the gastrointestinal tract

Enteroaggregative *E. coli* (EAEC) a pathotype of E. coli associated with acute and chronic diarrhea that aggregate to form microcolonies of adherent bacteria on the intestinal mucosa, where they release toxins (enterotoxins, Shiga-like toxin, EAST-1) that damage host cells and cause inflammation and diarrhea

Enterococci Gram-positive cocci once considered members of group D streptococci but now part of the genus *Enterococcus*; commensal species that have emerged as opportunistic pathogens

Enterocolitis inflammation of the gastrointestinal tract (enteritis in the small intestine, colitis in the colon) caused by a variety of microbial infections

Enterocyte attachment and effacement (EAE) altered ultrastructure of the apical surfaces of mucosal cells caused by proteins produced by EPEC

Enterohemorrhagic *Escherichia coli* (EHEC; *Escherichia coli* O157:H7) strains of *E. coli* that cause dysentery-like disease but rarely invade host cells; produce Shiga toxin; may cause hemolytic uremic syndrome

Enteroinvasive *Escherichia coli* (EIEC) strains of *E. coli* that cause invasive disease similar to that caused by *Shigella* but do not produce Shiga toxin and do not cause hemolytic uremic syndrome

Enteropathogenic *Escherichia coli* (EPEC) strains of *E. coli* that produce ultrastructural changes in mucosal cells of the small intestine and cause infant diarrhea

Enterotoxigenic *Escherichia coli* (ETEC) strains of *E. coli* that produce two toxins, a cholera-like toxin (heat-labile toxin [HLT]) and a peptide hormone-like toxin (heat-stable toxin [HST])

Enterotoxin an exotoxin that acts specifically on the intestinal mucosa

Entrez an integrated search and retrieval system used by the National Center for Biotechnology Information (NCBI) for assembling data from major life science databases, including literature sources, sequences, and many other databases; the combined information is available at http://www.ncbi.nlm_nih.gov/sites/gquery

Enzyme-linked immunosorbent assay (direct ELISA, also indirect/sandwich ELISA) a sensitive and specific method for measuring the concentration of antigen in an unknown sample; in a direct ELISA, an antibody that binds to an antigen of interest also is linked to an enzyme or fluorescent protein or dye that can be used for detection; in an indirect or sandwich ELISA, the antigen of interest is bound between two antibodies: the capture antibody that is immobilized on a matrix and binds to the antigen of interest and the detection antibody that also recognizes the antigen and is linked to an enzyme that can be used in a colorimetric or fluorescence reaction to detect the binding to the captured antigen

Eosinophil type of granulocyte derived from bone marrow that contains granules, which can release proinflammatory cytokines; important responders against parasitic helminth infections; mediators of allergies, asthma and autoimmunity

EPEC see **enteropathogenic *Escherichia coli***

EPS see **extracellular polysaccharide**

Epidemic a disease that appears sporadically and affects many individuals in a community

Epidemiology the study of the distribution, transmission, and factors contributing to the health and disease conditions of human and/or animal populations; the study of the history and emergence and spread of infectious diseases

Epidermis the outermost layer of the skin

Epithelia (singular epithelium) the layers of cells that cover the surfaces of the body and body cavities

Epitope a portion of an antigen recognized by an antibody-binding site; usually 5 to 9 amino acids in length for protein antigens

Epitope-based targeting signals a technique used to enhance dendritic cell binding properties to enhance immune responses and antigen presentation

Epizootic disease (also epizoonosis) a disease or disease outbreak that is prevalent or widespread (then, also referred to as panzootic) in an animal population

Epsilon-toxin a diarrhea-inducing exotoxin produced by *Clostridium perfringens*

Eradication complete reduction of the prevalence of an infectious disease in the global host population to zero; complete removal of all cases of a particular disease from everywhere

erm antibiotic resistance genes conferring resistance to macrolides, streptogramins, and lincosamides

Erythema redness or rash of the skin due to dilation of blood vessels

Erythromycin a member of the macrolide family of antibiotics

Eschar a black necrotic lesion surrounded by edema; characteristic of cutaneous anthrax

Escherichia coli* O157:H7** see **enterohemorrhagic *Escherichia coli

ESKAPE pathogen one of six bacterial pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) commonly associated with antibiotic resistance, often multidrug resistant (MDR), that are the leading causes of nosocomial infections throughout the world; an acronym for their names that also reflects their ability to escape antibiotic treatment

ETA exfoliative toxin of *Staphylococcus aureus* that cleaves desmoglein-1 of epidermal cells

ETEC see **enterotoxigenic *Escherichia coli***

ETs see **exfoliative toxins**

Eukaryotes organisms in which DNA is enclosed in a nuclear membrane

European Committee on Antimicrobial Susceptibility Testing (EUCAST) a European regulatory committee that establishes guidelines, standards and calibrations for minimal inhibitory concentration (MIC) breakpoints, zones of inhibition, and other technical aspects of phenotypic in vitro antimicrobial susceptibility testing

Exfoliation sloughing of cells

Exfoliative toxins (ETs) dermolytic exotoxins produced by *Staphylococcus aureus;* cause symptoms of scalded-skin syndrome and bullous impetigo; one type cleaves desmoglein-1, which holds keratinocytes together

ExoA see **exotoxin A**

Exoenzymes toxins introduced directly into host cells by specialized secretion systems; also called effector proteins

Exofoliative toxin a family of exotoxins with serine protease activity that are produced by Staphylococcus aureus and cause bullous impetigo, blistering of the skin, called scalded skin syndrome; hydrolyze desmosome proteins in the epithelial layer

Exotoxin a protein toxin produced by bacteria; usually secreted into the extracellular medium

Exotoxin A (ExoA) an AB protein toxin produced by *Pseudomonas aeruginosa;* it has the same mechanism of action as diphtheria toxin

ExPortal a membrane microdomain in *Streptococcus pyogenes;* may coordinate interactions between proteins secreted by the Sec system and membrane-associated chaperones

Extended-spectrum β-lactamases (ESBLs) a group of plasmid-encoded β-lactamases that confer antibiotic resistance to 3rd generation, extended-spectrum cephalosporins with broad range of antibiotic activity against many Gram-negative and Gram-positive bacteria

Extensively drug resistant (XDR, also extensive drug resistance) strains of bacteria that are resistant to at least three different classes of antibiotics; defined as nonsusceptibility to at least one drug in all but two or fewer antibiotic categories

Extracellular matrix (ECM) extracellular protein-polysaccharide material in which mammalian cells are embedded; contains collagen, hyaluronic acid, elastin, etc.

Extracellular polysaccharide secreted polysaccharides that form a major component of biofilms

Extravasation see **transmigration**

Exudate fluid and cells that have escaped from blood vessels

F factor (fertility factor, also called the F plasmid) a conjugative plasmid that allows genes to be transferred from a bacterium carrying the factor to another bacterium lacking the factor by conjugation

Fab the portion of an antibody that contains a light chain and the amino-terminal part of the heavy chain; the portion that contains the antigen-binding sites

Factor B a serum complement component protein that is cleaved into Ba and Bb by factor D

Factor D a serum complement component protein that cleaves factor B into Ba and Bb

Factor H a serum complement component protein that binds with sialic acid residues on host cells and activates C3; C3b is formed but remains bound to H; targets degradation of C3b

Factor I a serum complement component protein that degrades C3b bound to factor H

Facultative bacteria that can use either fermentation or respiration to obtain energy, depending on whether oxygen is present

Fc the portion of an antibody that binds complement and attaches the antibody-antigen complex to phagocytes; mediates opsonization; region of antibody bound by some streptococcal and staphylococcal surface proteins

Fc receptors a surface protein receptor on phagocytic immune cells that recognizes the Fc region of antigen-bound antibodies and mediates opsonization of the antigen-antibody complex

Fe utilization regulator see **ferric uptake regulator**

Febrile having a fever (elevated temperature)

Fecal microbiota transplantation (FMT) a therapy used to prevent clostridial-mediated enterocolitis disease, where stool from a healthy donor containing fecal microbiota, made into a liquid mixture, is transferred to the colon of a patient to restore the gut microbiome homeostasis

Fenton reaction the nonenzymatic formation of hydroxyl radical from iron and hydrogen peroxide

Ferric uptake regulator (Fur) a repressor protein that controls iron-regulated genes in many gram-negative bacteria

Ferritin intracellular iron storage protein of mammalian cells

fha see **filamentous hemagglutinin**

Fha see **filamentous hemagglutinin**

Fibrin a fibrous protein involved in the formation of blood clots

Fibrinolysis the enzymatic breakdown/cleavage of fibrin in blood clots, normally by plasmin, but can also be caused by bacterial virulence factors with protease activity

Fibronectin a high-molecular-mass (~440-kDa) extracellular matrix glycoprotein found on the surfaces of many host cells that binds to membrane-spanning receptor proteins (integrins)

Fibronectin-binding proteins (FnBP) staphylococcal and streptococcal surface proteins that bind fibronectin

Ficolins (FCNs) a group of oligomeric lectins with subunits consisting of collagen-like sequences and fibrinogen-like globular domains with lectin activity (usually bind to N-acetylglucosamine [GlcNAc]), secreted pattern-recognition receptors that can activate the lectin pathway of the complement cascade

Filament (i) a thin thread-like fiber made of a chain of tandem protein subunits located within a cell (such as myofilament, microfilament) or outside a cell (such as pilus or flagella); (ii) elongation of individual bacterial cells without septa formation or cell division, even though may have multiple chromosomes

Filamentous hemagglutinin (FHA) an adhesin produced by *Bordetella pertussis*; often a component of acellular pertussis vaccines

Fim see **fimbriae**

Fimbriae (Fim) short thin fibrils on the surfaces of bacteria; pili

Firefly luciferase (*luc*) see **Bioluminescence**

FITC (fluorescein isothiocyanate) a green fluorescent dye

Flagella rod-like protein structures projecting from bacterial cells; responsible for motility

Flagellin the main structural protein constituting flagella; a TLR agonist

Flavohemoglobin an enzyme that converts nitric oxide (NO) to NO^{3-} using heme-bound O$_2$; resistance mechanism found in *Escherichia coli*

Flora (also microflora) see **microbiota**

Fluorescein isothiocyanate see **FITC**

Fluorescence resonance energy transfer (FRET, also Förster resonance energy transfer) energy transfer between two light-sensitive molecules (chromophores/fluorophores), where a donor chromophore in its electronic excited state transfers energy to an acceptor chromophore through dipole-dipole coupling interactions; in fluorescence microscopy, FRET efficiency of energy transfer is measured to determine interactions between fluorescently labeled molecules

Fluoroquinolone a member of the quinolone family of antibiotics

FnBP see **fibronectin-binding proteins**

Follicles patches of tissue found in intestine; contain cells of GALT

Food and Drug Administration (FDA) a federal agency of the United States Department of Health and Human Services that is responsible for protecting public health by ensuring the safety, efficacy, and security of human and veterinary drugs, biological products and medical devices; by ensuring the safety of the food supply, cosmetics and products that emit radiation; by regulating tobacco products; by providing accurate, science-based information about food and health products; and, by providing counterterrorism capability

Foodborne bacterial disease a disease acquired by ingesting food contaminated with bacteria

Foodborne infection an infection acquired by ingesting food contaminated with microbes

Food botulism (also foodborne botulism) botulism (see also **botulism**) caused by ingestion of food containing botulinum neurotoxin or botulinum neurotoxin-producing bacteria such as *Clostridium botulinum*

Food irradiation technology ionizing radiation techniques for irradiating food at the last stages of processing to eliminate microorganisms

FoodNet a surveillance program to monitor cases of infection by foodborne pathogens, e.g., *Salmonella* and *Campylobacter*

Food poisoning illness caused by ingestion of food contaminated with pathogenic bacteria, viruses, parasites or toxins

Fosfomycin an antibiotic that blocks conversion of UDP-NAG to UDP-NAM during peptidoglycan synthesis

Fragilysin (or BFT, *Bacteroides fragilis* toxin) a zinc-dependent metalloprotease that is secreted by enterotoxigenic Bacteroides fragilis strains and cleaves E-cadherin to alter tight junction function in polarized intestinal epithelial cells; contributes to colitis and intestinal malignancy

Fragipain (Fpn) a cysteine protease produced by *Bacteroides fragilis* that cleaves and activates *B. fragilis* toxin (BFT)

Freund's complete adjuvant a mixture of oil and other components, including mycobacterial cell walls; used to stimulate antibody response to protein antigens

Freund's incomplete adjuvant the same as Freund's complete adjuvant minus the mycobacterial components

FtsY bacterial signal-recognition particle receptor that interacts with the SecYEG translocon of the General Secretory Pathway

Fur see **ferric uptake regulator**

Fura-2 a fluorescent dye reagent used to detect calcium levels inside tissue culture cells

Furin a ubiquitous, membrane-associated processing enzyme localized on the host cell membrane surface and in the trans-Golgi network; a subtilisin-like protease that cleaves protein precursors (including many bacterial protein toxins) to activate them

Fv see **variable region**

Galectin-8 a β-galactoside-binding lectin with conserved carbohydrate recognition domains involved in integrin interactions, cell-cell adhesion, and binding to damaged bacterial-containing vacuoles, which triggers recruitment of autophagy adaptors, such as NDP52, to cause formation of an autophagosome for bacterial killing

GALT see **gastrointestinal-associated lymphoid tissue**

Gamma interferon (IFN-γ) a cytokine that stimulates monocytes and PMNs to leave the bloodstream and stimulates endothelial cells to produce selectins

Gamma (γ) rays high-energy photons produced by radioactive material (usually cobalt-60 or cesium-137); used to irradiate food and sterilize medical equipment and supplies

Gamma-delta (γδ)T cells immune cells that may play a role in limiting the growth of intracellular pathogens

γ-Phage a lysogenic phage closely related to β- and ω-phages; does not encode diphtheria toxin

γ-Toxin a membrane-damaging exotoxin produced by *Clostridium perfringens*

Gangrene death of body tissue (necrosis) usually associated with loss of blood supply caused by bacterial invasion, bacterial toxins, putrefaction, or other diseases that cause cell death

GAS group A streptococci (e.g., *Streptococcus pyogenes*)

Gas chromatography a type of chromatography used in analytical chemistry for separating and analyzing compounds that can be vaporized without decomposition; often coupled with mass spectrometer for subsequent analysis and identification of the separated molecules

Gastritis inflammation of the stomach lining; can be caused by *Helicobacter pylori* infection in some individuals

Gastrointestinal-associated lymphoid tissue (GALT) the intestinal mucosal immune system characterized by production of secretory IgA (sIgA); a component of mucosa-associated lymphoid tissue (MALT)

GBS group B streptococci (e.g., *Streptococcus agalactiae*)

GenBank (National Center for Biotechnology Information [NCBI]) U.S. centralized library of various biological data, including nucleotide sequences

Gene amplification a process by which a single copy of a gene is replaced by multiple copies (e.g., genes imparting antibiotic resistance); can be through homologous recombination or through PCR

Gene chip see **DNA chip**

Gene conversion a process by which genetic information is transferred by a nonreciprocal mechanism; a process of pilin gene recombination responsible for antigenic variation in *Neisseria*

Gene duplication and amplification (GDA) repeated gene duplication events through non-equal homologous recombination between direct repeats or through RecA-independent recombination allows for amplification of the gene

Gene editing technology (also gene editing, genome editing, genomic engineering) a type of genetic engineering technology where DNA is inserted, deleted, modified/mutated, or replaced in the genome of an organism at precise locations; see also CRISPR-Cas9 systems, ZFN, and TALEN

General secretory pathway (Sec system) a common secretion pathway shared by both gram-negative and gram-positive bacteria through which many proteins are exported from the cytoplasm; requires an N-terminal signal sequence on the protein being secreted

Generalized transducing phage a type of phage that occasionally packages bacterial genomic DNA into the viral capsid and, following infection, transfers the DNA fragments into a new bacterial cell, where the new DNA is integrated into the bacterial DNA of the recipient cell by homologous recombination

Gene silencing (i) regulation of gene expression in a cell by preventing transcription or translation; (ii) an epigenetic modification of DNA that leads to inactivation of a previously active gene

Genetic ablation a method of modifying a specific gene to prevent gene expression; a form of gene silencing that can lead to killing and ablation of a particular type of cell

Genetical genomics a tool used to elucidate the basis of complex traits and disease susceptibility; statistical modeling of expression of quantitative trait loci (QTLs) that link genetic variation to corresponding phenotypic outcome

Genome the complete set of genes of an organism

Genome editing (also genetic engineering technology) see **Gene editing technology**

Genome sequence the order of nucleotide bases in the genome

Genome sequencing see **DNA sequencing technologies**

Genome-wide association studies (GWAS, also whole-genome association study, multi-locus trait analysis, MLTA) hypothesis-free methods to analyze genomic data identify associations or linkages between genetic regions (loci) and traits (such as diseases); a genomic approach to rapidly scan for biomarkers across many individuals to find genetic variations associated with a particular disease

Genomic islands regions of a genome, coding for a variety of different functions involved in metabolism, symbiosis, pathogenesis and/or adaptation, that has evidence suggesting that it was acquired through horizontal transfer

Genomic plasticity variability seen in the genome; caused by numerous mechanisms

Genomic subtractive hybridization (GSH) a PCR-supported method for isolating genomic DNA sequences unique to specific strains of closely related bacteria

Genomics a field of molecular biology that focuses on the study of the structure, function, evolution, gene mapping, editing and comparing of genomes

Genomics-based discovery identification of new targets for antibacterial drug discovery by comparing the genomes of a variety of pathogens for unique genes

Genotoxin a chemical or biological agent that causes damage to the genetic information of a cell that results in mutation, often subsequently leading to cancer

Genotype the genetic constitution of an organism that produces a certain phenotype

Gentamicin an antibiotic in the aminoglycoside family

Gentamicin protection assay a cell-based infection assay that permits distinction between bacteria that attach to the surfaces of host cells and those that invade the cytoplasm of the host cell

Germ-free animals see **gnotobiotic animals**

Germ-free (GF) see **gnotobiotic animals**

Germ theory of infectious disease the idea that there is a connection between a microbe and a particular set of disease symptoms

Germination the conversion of a bacterium from a dormant spore to a metabolically active, vegetative state

GFP see **green fluorescent protein**

Gingivitis (also periodontal disease) a form of gum disease caused by inflammation of the gums, often due to infection of dental plaque, a build-up of bacterial biofilm; can lead to tooth decay and bone loss

Glassification a procedure used to stabilize a vaccine by lyophilization of the protein antigen in the presence of sugar-containing stabilizers, such as trehalose

Global Alliance for Vaccines and Immunization (GAVI) an international organization with a mission to increase access to immunization, improve education about immunizations and benefits of vaccines to undereducated populations

Global regulator a single regulator that controls the expression of a large number and many types of genes involved in a broad range of physiological processes

Glycopeptide antibiotics a group of antibiotics that contain carbohydrate groups (glycans) covalently attached to the side chains of amino acid residues and that inhibit peptidoglycan synthesis; includes vancomycin and teicoplanin

Glycopeptides a group of antibiotics that inhibit peptidoglycan synthesis; includes vancomycin and teicoplanin

Glycosaminoglycans (GAGs, also mucopolysaccharides) a family of long, often sulfated, unbranched polysaccharides consisting of repeating disaccharide units of an amino sugar (N-acetylglucosamine or N-acetylgalactosamine) and an uronic sugar (glucuronic acid or iduronic acid) or galactose; a major component of the extracellular matrix in animal tissues

Glycosyltransferases a variety of enzymes that catalyze the formation of a glycosidic linkage to form a glycoside; enzymes that catalyze the transfer of sugar groups from an activated nucleotide sugar phosphates (the glycosyl donor) to a nucleophilic group (usually a hydroxyl, amine, or sulfuryl group) on the acceptor molecule

Glycyl-glycine tetracycline see **tigecycline**

GM-CSF see **granulocyte-macrophage** colony-stimulating factor

Gnotobiotic animal an animal raised in a sterile environment that has no bacteria in or on them; used for infection models of diseases

Goblet cells glandular simple columnar epithelial cells that secrete mucin

Golgi system an intracellular organelle that processes proteins destined for excretion from mammalian cells and determines what route they take to their ultimate destination

Granulocyte a lymphocyte with secretory granules in its cytoplasm; a neutrophil, basophil, or eosinophil

Granulocyte-macrophage colony-stimulating factor (GM-CSF) a cytokine that triggers release of monocytes and PMNs from bone marrow into the bloodstream

Granuloma a special type of inflammatory lesion or mass of immune cells containing actively growing fibroblasts, macrophages, and lymphocytes that forms when the immune system tries to wall off foreign substances that it cannot remove by other means

Granulosome a cytolytic granule found in phagocytes, cytotoxic T cells (CTLs), and natural killer (NK) cells that shares properties with lysosomes; contains myeloperoxidase, perforin (pore-forming cytolysin), and granzymes

Granulysin a cytolytic pore-forming protein released by CD8$^+$ cytotoxic T cells (CTLs) and natural killer (NK) cells that are attached to host cells infected with intracellular pathogens and presenting antigens through the MHC I complex

Granzymes a set of serine proteases produced and released by CD8$^+$ cytotoxic T cells (CTLs) and natural killer (NK) cells that enter host cells infected with intracellular pathogens through pores created by perforin and then initiate apoptosis (programmed cell death) by cleaving caspases

Gray (Gy) a unit of ionizing radiation dose that is defined as the absorption of one joule of radiation energy per kilogram of matter (J kg^{-1})

Green fluorescent protein (GFP) a green fluorescent protein (encoded by modifications to the *gfp* gene originally from a jellyfish) used as a reporter for gene transcription and when fused with a target protein as a marker for intracellular localization using fluorescence microscopy

Group A streptococci a designation of *Streptococcus pyogenes* based on surface carbohydrate antigen, according to the Lancefield grouping system

Group B streptococci a designation of *Streptococcus agalactiae* based on surface carbohydrate antigen, according to the Lancefield grouping system

GUS see **β-glucuronidase**

H antigen bacterial flagellar antigen recognized by antibodies; used to identify bacterial serotypes, especially in *Escherichia coli* and related species

HA (also hospital associated, hospital acquired) see **nosocomial infection**

HACCP see **hazard analysis and critical control point**

Halides disinfectants, such as chlorine and iodine, that oxidize and inactivate bacterial proteins

Hamamelitannin a small molecule isolated from witch hazel bark that inhibits the quorum-sensing regulator of methicillin-resistant *Staphylococcus aureus* (MRSA)

Hazard analysis and critical control point (HACCP) a food and drug safety management system based on a surveillance program to identify steps in the food- and drug-processing stream where contamination might occur

HB-EGF see **heparin-binding epidermal growth factor**

Heat shock proteins a class of proteins whose expression is increased by stress; can enhance immune response

Heat-labile toxin (HLT) a multisubunit AB_5 exotoxin produced by ETEC; similar to cholera toxin in mechanism of action and sequence

Heat-stable toxin (HST) a peptide hormone-like diarrheal toxin produced by ETEC; STIa mimics guanylin, an intestinal hormone that increases intracellular cyclic GMP levels

Heavy chain the larger of two types of proteins making up an antibody molecule; the receptor-binding and translocation domains of botulinum neurotoxins

Hemagglutination clumping together of red blood cells (RBCs)

Hematogenous produced by, derived from, or spread by blood

Heme a group of porphyrin-like proteins that tightly bind to iron; includes metalloproteins, such as peroxidases and electron transfer proteins, and hemoglobin

Hemoglobin a heme-containing protein in the human body that binds iron

Hemolysin (Hly) a pore-forming protein toxin that causes lysis of erythrocytes (red blood cells)

Hemolytic-uremic syndrome (HUS) acute irreversible kidney failure caused by Shiga toxin, produced by *Shigella* strains, and Shiga-like toxins, produced by STEC (e.g., *Escherichia coli* O157:H7) and EHEC (enterohemorrhagic *E. coli*) strains; characterized by hemolytic anemia, acute renal (kidney) failure, low blood platelet count, and possibly death

Hemorrhage (also hemorrhaging, bleeding) escape or leakage of blood (bleeding) from ruptured blood vessel

Heparin an anticoagulant produced by basophils and Mast cells that prevents blood clotting

Heparin-binding epidermal growth factor (HB-EGF) a protein that is involved in wound healing and cardiac hypertrophy; the mammalian cell-bound precursor serves as the cellular receptor for diphtheria toxin

Hepatotoxin a bacterial toxin that targets liver cells

Herd immunity protection of unvaccinated people in a population where most people are vaccinated

Hfq bacterial protein that binds to regulatory small RNA (sRNA) and acts as a chaperone to stabilize the sRNA and/or mediate base pairing with mRNA to regulate translation

Hfq-dependent sRNAs in gram-negative bacteria, the largest class of small RNAs (sRNAs) that use Hfq protein as a chaperone to facilitate base pairing between sRNA and mRNA targets

HGT see **horizontal gene transfer**

Hib a conjugate vaccine against *Haemophilus influenzae* type b; consists of a polysaccharide capsule attached covalently to a protein (tetanus toxoid, diphtheria toxoid, or meningococcal group B outer membrane protein)

High-frequency recombination cell (Hfr) a bacterial cell with an episome, a conjugative plasmid (such as the F-factor) integrated into its chromosome through homologous recombination, which is able to efficiently deliver chromosomal genes into recipient cell (F^-) that lacks the episome

High mobility group box 1 (HMGB1) protein an intracellular protein that can translocate to the nucleus where is acts as chromatin protein and interacts with nucleosomes, transcription factors and histones to organize DNA and regulate transcription; secreted by activated immune cells as an alarmin cytokine mediator of inflammation

High-performance liquid chromatography (HPLC) a form of column chromatography in which a pump is used to move material through a densely packed column, resulting in high resolution of the compounds being separated

High-throughput screening (HTS) an approach for scientific discovery that uses automated processing (robotics) or combinatorial libraries to screen massive numbers of compounds or to perform large numbers of assays or reactions (usually in 96-well or 384-well microplates) to identify active compounds, drugs, mutants, or other targeted products with the desired properties

Histamine a vasoactive compound released by basophils and mast cells to trigger an inflammatory response by increasing capillary permeability to white blood cells to allow them access to the site of infection

Histatins antimicrobial peptides, produced by host cells, that are secreted in saliva

Histidine kinase the signal-transducing protein in bacterial two-component regulatory systems that autophosphorylates on a histidine residue in response to an environmental condition or signal and transfers the phosphoryl group to an aspartate residue on its cognate response regulator; also called sensor kinase

HLT see **heat-labile toxin**

HME see **human monocytotropic ehrlichiosis**

HNH protein (also HNH domain) a bacteriophage endonuclease that is involved in phage DNA packaging into the capsid of the phage; an endonuclease domain of the CRIPR-associated protein Cas9 that mediates cleavage of the target strand of DNA

H-NS a histone-like (nucleoid-associated DNA-binding) protein that is a global repressor of transcription in gram-negative bacteria; represses transcription of foreign genes acquired by horizontal gene transfer

HO• (also •OH) see **hydroxyl radical**

Holins phage proteins that form pores in bacterial membranes

Homology-directed repair (HDR) a common form of homologous recombination used in cells to repair double-stranded DNA lesions, which relies on a homologous DNA sequence as a template for restoring the correct sequence

Horizontal gene transfer (HGT) lateral movement of a group of genes from one bacterium to another through various mechanisms, including conjugation, transduction, and transposition

Hospital acquired infection (HA, also hospital associated infection) see **nosocomial infection**

Hospital associated infection (HA, also hospital acquired infection) see **nosocomial infection**

Host (i) a human or animal body in which a resident microbiota lives; (ii) a human or animal body colonized and attacked by bacterial pathogens; (iii) in the context of bacterial viruses, the bacterium that is attacked by bacteriophage

Host-microbe relationship (also host-pathogen interaction) the interplay between a host and a microbe or pathogen at the molecular, cellular, organismal and populational level that defines how a microbe survives within the host and/or causes infection

Host-parasite interaction the relationship between the human or animal body (host) and the invading bacterium (parasite)

HPLC see **high-performance liquid chromatography**

HST see **heat-stable toxin**

HTS see **high-throughput screening**

Human immunodeficiency virus (HIV, also HIV-1, HIV-2) a lentivirus (retrovirus, single-stranded, positive-sense, enveloped RNA virus) that infects human CD4$^+$ T cells, converts its RNA genome in DNA and integrates into the host genome; can lead to acquired immunodeficiency syndrome (AIDS) due to progressive deterioration of the immune system

Humanized animal (also humanized transgenic animal) a transgenic animal, such as a mouse, that carries functional human genes, cells, tissues and/or organs; commonly used as animal infection models to more closely mimic the human disease

Human Microbiome Project (HMP) a massive research initiative launched in 2007 by the United States National Institutes of Health to improve the understanding of the microbiota involved in human health and disease, with the first phase focused on identifying and characterizing the microbial communities of human body sites and the second phase aimed at characterizing the roles of the microbiomes in healthy and disease states

Human monocytotropic ehrlichiosis (HME) potentially fatal febrile illness due to *Ehrlichia* species infecting monocytes

Human umbilical vein endothelial cells (HUVEC) primary human endothelial cells, obtained from the umbilical vein, used to study bacterial transit across the endothelium and to study macromolecule transport, blood coagulation, fibrinolysis, immune cytokine responses, and cell adhesion

Humoral immunity adaptive, MHC II-dependent, CD4$^+$-dependent, antibody-mediated immunity

HUS see **hemolytic-uremic syndrome**

HUVEC see **human umbilical vein endothelial cells**

Hyaluronate lyase (Hyl) an enzyme produced by *Streptococcus pneumoniae* and other bacterial pathogens that dissolves hyaluronic acid, thereby promoting bacterial spread and providing food for the bacteria

Hyaluronic acid an anionic, nonsulfated glycosaminoglycan (mucopolysaccharide) widely distributed throughout connective, epithelial, and neural tissues; a major component of the extracellular matrix; also a component of the group A streptococcal extracellular capsule that is important for immune evasion

Hyaluronidase an enzyme that degrades hyaluronic acid

Hydrogen peroxide (H2O2) a reactive oxygen compound produced by metabolism that acts by oxidizing proteins and damaging DNA; used as an external antiseptic

Hydroxamate a class of siderophores

Hydroxyl radical (HO•, •OH) a reactive form of oxygen that can kill bacteria

Hyl see **hyaluronate lyase**

Hypermucoviscous a phenotype of certain hypervirulent variants of bacterial pathogens, such as Klebsiella pneumoniae, that are hypervirulent and produce excessive amounts of extracellular polysaccharide capsule with a characteristic highly viscous, stringy colony phenotype

Hypermutable having higher-than-normal mutation rates

Hypochlorite a reactive form of chlorine toxic to bacteria

Hypotension low blood pressure (with a systolic to diastolic ratio < 90/60 mmHg), which can cause fainting or dizziness, often a consequence of septic shock or toxic shock

Hypothiocyanite (OSCN$^-$) a reactive form of thiocyanate that is highly toxic to bacteria; oxidation of thiocyanate by hydrogen peroxide into hypothiocyanite is catalyzed by lactoperoxidase

ICAM see **intercellular adhesion molecule**

iC3b factor I-catalyzed proteolytic digestion product of C3b that opsonizes but does not form C3 convertase

ID50 value (also 50% Infectious dose) the number of microorganisms required to cause infection (as evidenced by a clearly defined symptom) in 50% of experimentally infected animals at a given time following infection; a measure of infectivity

IEL see **intestinal epithelial lymphocytes**

IFN-γ see **gamma interferon**

IgA (also sIgA) see **immunoglobulin A**

IgE see **immunoglobulin E**

IgG see **immunoglobulin G**

IgG1 to IgG4 see **immunoglobulin G**

IgM see **immunoglobulin M**

IL-1 see **interleukin 1**

IL-1 receptor antagonist (IL-1ra) an anti-inflammatory cytokine

IL-2 see **interleukin 2**

IL-3 see **interleukin 3**

IL-4 see **interleukin 4**

IL-6 see **interleukins 6**

IL-8 see **interleukin 8**

IL-10 see **interleukin 10**

IL-13 see **interleukin 13**

Illumina RNA-Seq technology see **RNA-Seq technology**

Illumina sequencing technology see **DNA sequencing technologies**

Illumina Tn-Seq technology see **Tn-Seq technology**

ILs see **interleukins**

Imipenem a β-lactam antibiotic

Immune potentiators (also adjuvants, augmenters) see **adjuvants**

Immune system a complex collection of phagocytic, cytotoxic, and antibody-producing cells and protein components that protect the body from infection

Immunofluorescence a fluorescence microscopic technique used to directly or indirectly detect cellular antigens by using antigen-specific antibodies that have been conjugated with fluorescent dyes

Immunogen an antigen that induces an immune response

Immunogenic the ability to elicit a robust antibody or T cell response

Immunoglobulin A (IgA, also secretory immunoglobulin A, sIgA) the major class of antibody found in mucosal tissues; produced as a dimer and secreted into the lumen as sIgA; sIgA, the dimeric form of IgA with two IgA molecules bound by a piece of the receptor protein (secretory piece) that is found in luminal secretions; provides local immune protection for mucosal membranes by binding both mucin and bacteria, which are then sloughed off

Immunoglobulin E (IgE) a class of monomeric antibody thought to play a role in control of metazoan and protozoan parasites; involved in allergic responses

Immunoglobulin G (IgG, also IgG1 to IgG4) the major monomeric antibody class present in the serum and tissue fluids; produced by plasma B cells and binds to many kinds of pathogens to cause agglutination, immobilization, activation of the classical complement pathway, opsonization for phagocytosis, and neutralization of bacterial toxins; important for antibody-dependent cell-mediated cytotoxicity (ADCC); IgG1 to IgG4, subtypes of human IgG

Immunoglobulin M (IgM) the first class of multimeric (usually pentameric) antibody produced by activated B cells in response to an antigen; possesses high avidity for antigen and is very effective at complement activation

Immunoglobulins antibodies

Immunohistochemistry a histological technique used to detect bacteria in tissue samples that uses labeled antibodies against the bacteria to visualize the bacteria through microscopy

Immunomodulation regulatory adjustment or modification of the immune system to elicit a particular response

Immunoprecipitation a technique to precipitate a protein antigen out of solution by using an antibody specific for that protein; the antibody is usually coupled to a solid substrate, such as a bead

Immunoproteomics a proteomic technique for identifying bacterial antigens that elicit distinct host responses

Immunosuppression suppression (dampening) of the normal immune response

Immunotoxins hybrid proteins containing the catalytic cytotoxic domain of a toxin (such as the A fragment of DT) and a cell-targeting antibody or receptor ligand that recognizes a specific host cell type; used as a basis for new therapeutic approaches against cancer cells or virus-infected cells

In vitro an environment outside the body (usually a test tube)

In vivo inside the body, as in an animal model of infection

In vivo expression technology (IVET) a high-throughput screening approach for identifying bacterial genes that are expressed only when the bacteria are in the host

In vivo infection model an animal model of disease used to study colonization, invasion, and establishment of infection and pathogenesis caused by pathogens

In vivo-induced antigen technology (IVIAT) an antibody-based genomic method used to identify genes induced during human infections that uses antibodies present in the sera of convalescent patients to identify antigens of the pathogen that were expressed during infection and that elicited an immune response

IND see **investigational new drug**

Indels spontaneous DNA mutations resulting from insertions or deletions

Indication a medical sign or symptom that is a valid reason to use a certain intervention or treatment modality (test, medication, procedure or surgery)

Indirect ELISA see **Enzyme-linked immunosorbent assay**

Indolyl-galactoside see **X-Gal**

Induced tolerance see **anergy**

Infant botulism flaccid paralysis that results when botulinum neurotoxin is produced by *Clostridium botulinum* colonizing the colon of an infant

Infection the successful colonization of the body by a microorganism capable of causing damage to the body (i.e., a pathogen)

Infectious capable of causing disease

Inflammation an immune response of the body to irritants, such as infection by a pathogen; characterized by redness, swelling, pain, and heat

Inflammatory bowel disease (IBD) a chronic inflammation of the intestinal tract that manifests as two diseases: ulcerative colitis and Crohn's Disease (CD)

Inflammatory response see **inflammation**

Inflammasome an intracellular multiprotein complex of immune NOD-like receptors and sensors that detects the presence of intracellular microbes, activates caspase-1, and releases the highly proinflammatory cytokines, IL-1 and IL-18

Injectisome the delivery apparatus of the type 3 and type 4 secretory systems, consisting of a complex of many proteins, that directly injects the effector proteins into the host cell

InlA see **internalin A**

InlB see **internalin B**

Innate immune system the host defenses that are always present and effective against most bacteria; includes physical barriers, complement, phagocytic cells, and the washing action of fluids

Inoculum the number of bacteria (in CFU) introduced into a culture medium, tissue culture, or animal model of infection

INSD see **International Nucleotide Sequence Database**

Insertion sequences sites flanking a transposon and consisting of inverted repeats that direct transposition (insertion) of the transposon via a transposase into the new regions of DNA

Institutional animal care and use committee (IACUC) a federally mandated committee of an institution, comprised of experienced experts in animal care and handling, that oversees the institution's animal programs, facilities and procedures to provide a framework for compliance with government policies, guidelines and biosafety practices related to the use of animals in research, teaching and testing

Institutional review board (IRB) a board made up of a diverse group of professionals at a given institution that must approve human clinical trials or research experiments involving human subjects

Integrase an enzyme encoded by a plasmid gene that mediates insertion of DNA (such as a plasmid) into the DNA of a recipient cell

Integrative conjugative element (ICE)

Integrative mobilizable elements (IMEs)

Integrins (also α-integrins or β-integrins) heterodimeric protein receptors (comprised of α and β subunits) on the surfaces of cells that mediate attachment between the cell and the surrounding tissues (cells or extracellular matrix); integrins on PMNs bind to ICAMs on endothelial cells; binding stops the movement of PMNs through the bloodstream

Integron an integrating element probably responsible for the evolution of plasmids carrying multiple antibiotic resistance genes

Intellectual property (IP) a term referring to inventions (such as antibiotics and drugs) for which patents are issued to protect the owner's property rights

Intercellular adhesion molecule (ICAM) an adhesion molecule of endothelial cells that binds to integrins on PMNs; aids PMN migration out of blood vessels

Intergenic see **intergenic recombination**

Intergenic recombination homologous recombination of genes from different bacterial cells

Interleukins 1 (IL-1) a family of potent proinflammatory cytokine immune signaling molecules produced by monocytes and macrophages; may contribute to septic shock symptoms; the IL-1 superfamily consists of 11 members with similar structures, most well-known members are IL-1α, IL-1β, IL-1Ra (antagonist for IL-1α and IL-1β), and IL-18

Interleukin 2 (IL-2) a cytokine immune signaling molecule produced by T cells that stimulates T cell proliferation

Interleukin 3 (IL-3) a cytokine immune signaling molecule that stimulates release of monocytes and PMNs from bone marrow into the bloodstream

Interleukin 4 (IL-4) a cytokine immune signaling molecule produced by T cells, mast cells and basophils that induces differentiation of naïve helper T cells (Th0 cells) into Th2 cells; Th2 cells produce more IL-4 to further stimulate proliferation of Th2 cells and B cells

Interleukins 6 (IL-6) a cytokine immune signaling molecule produced by monocytes and macrophages; acts as both proinflammatory cytokine and anti-inflammatory myokine (produced by muscles): as a proinflammatory cytokine contributes to septic shock symptoms of fever and other acute phase responses; as myokine inhibits IL-1, IL-10 and TNFα

Interleukin 8 (IL-8) a chemotactic and inflammatory cytokine immune signaling molecule produced by macrophages and a variety of tissue and blood cells that stimulates monocytes and granulocytes to leave the bloodstream and move to a site of infection

Interleukin 10 (IL-10) an anti-inflammatory cytokine immune signaling molecule that inhibits the activation and effector function of T cells, monocytes, macrophages; its immunosuppressive properties dampen inflammation and autoimmune responses

Interleukin 13 (IL-13) an anti-inflammatory cytokine immune signaling molecule structurally similar to IL-4; secreted by Th2 cells, CD4 cells, NK cells, mast cells, basophils, and eosinophils; regulates IgE synthesis, mucus hypersecretion; associated with airway disease such as asthma

Interleukins (ILs) protein cytokines, immune signaling molecules produced and secreted by leukocytes, monocytes, dendritic cells, macrophages, T cells, B cells, and other immune cells, that mediate inflammatory responses (inflammation, septic shock, and fever); maturation, differentiation, and/or proliferation of immune cells; chemotaxis; angiogenesis; and production and release of cytokines and chemokines

Internalin A (InlA) an adhesin protein of *Listeria monocytogenes* that binds E-cadherin on host cells and facilitates adhesion and invasion

Internalin B (InlB) an adhesin protein of *Listeria monocytogenes* that binds the receptor tyrosine kinase Met on host cells and facilitates adhesion and invasion

International Nucleotide Sequence Database (INSD) large primary sequence databases consisting of NCBI, GenBank, EMBL NDB, DDBJ, UniProtKB, Swiss-ProtKB, and PDB RCSB

Intestinal epithelial lymphocytes (IELs) lymphocytes found in the epithelial layer of the mucosa such as GI and urogenital tracts; cytotoxic T cells ($CD8^+$, $CD3^+$) of the mucosal immune system that do not need priming and immediately release cytokines upon encountering antigens

Intragenic (also intragenomic) see **intragenic recombination**

Intragenic recombination (also intragenomic recombination) homologous recombination of genes within a single bacterium

Intranasal inoculation (i) instilling a vaccine in the nasal passages; activates the BALT; (ii) inoculating bacteria by inhalation into the nose in animal models; depending on the model, the bacteria colonize the nasopharynx or progress from the nasopharynx and cause invasive disease

Invasin a bacterial surface protein that provokes endocytic uptake by host cells

Invasion frequency the ratio of the CFUs of gentamicin-resistant intracellular bacteria to the CFUs of cell-associated bacteria (before gentamicin treatment) at the end of a gentamicin protection assay

Invasion success curve a method for determining the maximum number of internalized bacteria per host cell; used to compare pathogenic bacterial strains

Invasive capable of penetrating the host's defenses; capable of entering host cells or passing through mucosal surfaces and spreading in the body

Inversion of sequences a process by which bacteria invert the DNA sequence, such as a promoter upstream of a particular gene; could lead to phase variation or rearrangement of genes

Investigational new drug (IND) a promising new drug that must be tested in two animal models of infection to evaluate efficacy and safety; if the FDA approves the IND application for a particular drug candidate, the drug can enter a phase I clinical trial

IP see **intellectual property**

IRB see **institutional review board**

IS elements see **insertion sequences**

Ischemia a restriction in blood supply to tissues that causes a shortage of oxygen needed for cellular metabolism; usually results from damage or dysfunction as a result of infection or injury

Isoelectric focusing (IF) an electrophoretic technique that separates proteins based on their isoelectric point (pI)

Isoelectric point (pI) the pH at which a protein has no net charge and does not move in an electric field

Isolation membrane (or phagophore) a membrane fold that appears upon induction of autophagy that forms around the cytosolic components being sequestered to form a double-membrane vesicle, the autophagosome, which then delivers the components to the lysosome for degradation

IVIAT see **in vivo-induced antigen technology**

J chain a peptide linking IgA monomers or IgM monomers

JGI Genomics Department of Energy Joint Genome Institute; database for many eukaryotic and microbial genomes

K antigen the capsular antigen used for classification of *Escherichia coli*

K1 antigen a component of the antiphagocytic capsule of *Escherichia coli* strains that cause septicemia and meningitis

Kallidin a vasoactive peptide that enhances extravasation

Kanamycin an antibiotic in the aminoglycoside family

Keratinocyte a stratified squamous cell constituting up to 95% of the epidermis; maintains the acidic environment of the skin; forms the keratin layer that protects the skin and the underlying tissues from the external environment; produces keratin and cytokines; produces cytokines that modulate the immune response of the skin

Ketolides new derivatives of macrolide antibiotics

Keystone pathogen a low-abundance microbial pathogen that can orchestrate inflammatory disease by remodeling a normally benign or healthy microbiota into a dysbiotic microbiota that then induces diseases

Kirby-Bauer disc diffusion assay an antibiotic susceptibility test that uses antibiotic-infused disc to test the extent to which bacteria are prevented from growing near the disc on an agar plate; the size of the zone of clearing indicates the effectiveness of the antibiotic on a bacterium

Knock-in mice transgenic mice that have had human genes introduced into their genomes and express certain human proteins in infection models

Knockout mice transgenic mice that have disruptions in specific genes

Koch's postulates a set of postulates that must be met to prove a particular bacterial pathogen causes a particular disease; there are four postulates that must be satisfied, but today there are additional modern molecular methods for satisfying these postulates

Kupffer cell a resident (fixed) macrophage in the liver

L forms bacteria that lack a cell wall

Lactoferrin a mammalian host protein found in secretory fluids, such as milk, saliva, tears, and nasal secretions, that binds iron with high affinity

Lactoperoxidase a heme-iron-containing enzyme found in mucus that catalyzes the oxidation of inorganic and organic compounds (chloride, bromide, iodide, and thiocyanate) by hydrogen peroxide to form reactive oxygen species (hypochlorite, hypobromite, hypoiodite, and hypothiocyanite)

LacZ see **β-galactosidase**

***lacZ* gene** a gene that encodes β-galactosidase

Lamina propria a thin layer of connective tissue and fibrillar material embedded in a mucopolysaccharide matrix that is located beneath the epithelial layer of mucosa

Laminins high molecular weight (400-900 kDa) proteins of the extracellular matrix that are a major component of the basal lamina (one of the layers of the basement membrane)

Lancefield grouping system immunology-based classification of surface antigens used to identify and characterize *Streptococcus* species; devised by prominent microbiologist Rebecca Lancefield

Langerhans cells a type of antigen-presenting cell (dendritic cells) found in the epidermis (skin) and lymph nodes

Laser capture microdissection (LCM) a procedure for attaching isolated host cells to transparent parafilm by exposure to a low-energy near-infrared laser pulse, so that the nucleic acids or proteins can then be isolated from the attached cells

Latency a period of inactivity or dormancy

Lateral surface the side portion of polarized epithelial cells

LBP see **LPS-binding protein**

LC3 (microtubule-associated protein 1A/1B-light chain 3) see **Autophagy-related protein**

LCM see **laser capture microdissection**

LD50 value (50% Lethal dose) the number of bacteria (in CFU) or the amount of toxin required to kill 50% of the animals experimentally inoculated within a given period of time

Leader peptide a short regulatory peptide involved in transcriptional or translational attenuation mechanisms of gene control; encoded by transcripts from leader regions of operons controlled by attenuation (e.g., 100 bp upstream of the start codon for erythromycin resistance protein); leader peptides are not signal sequences

Leader region the upstream transcript region between the 5′ end of a transcript and the translation start site of the first structural gene; often encodes regulatory leader peptides

Leader sequence see **signal sequence**

Lecithin a phospholipid (e.g., phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol) component of host cell membranes

Lecithinase a phospholipase protein toxin produced by *Clostridium perfringens* or *Listeria monocytogenes* that disrupts host cell membranes

Lectin pathway one of the pathways that comprises the complement cascade and is initiated by the mannose-binding lectin (MBL), collectin or ficolin binding to the microbial antigen, and proceeds through the action of C4 and C2 to generate the C3 and C5 convertase complexes

LEE see **locus of enterocyte effacement**

Lethal factor (LF) the zinc-dependent metalloprotease subunit of anthrax toxin complex produced by *Bacillus anthracis*

Leukocidin an extracellular pore-forming toxin produced by *Staphylococcus aureus* that kills leukocytes

Leukocyte a white blood cell; cells of the immune system

Leukotoxin a pore-forming protein toxin produced by some bacteria that kills leukocytes

Light chain the smaller of two proteins making up the basic antibody structure; the catalytic-domain-containing portion of botulinum neurotoxins

Lincomycin a member of the lincosamide family of antibiotics

Lincosamides a family of antibiotics that binds to the 23S rRNA of the 50S ribosomal subunit

Linezolid a member of the oxazolidinone family of synthetic antibiotics

Linkage (also genetic linkage) two genes or genetic markers that are close to each other on a chromosome and so have a lower chance of recombination between them and are more likely to be inherited together

Lipid A (endotoxin) the toxic portion of lipopolysaccharide (LPS) from gram-negative bacteria that is embedded in the outer membrane of gram-negative bacteria

Lipinski's "Rule of Five" a "rule of thumb"' used by medicinal chemists to evaluate whether a potential new lead compound for a drug candidate is orally active without being toxic in humans

Lipooligosaccharide (LOS) a component of the outer membrane of some gram-negative bacteria (e.g., *Neisseria* and *Yersinia* species) that is similar to lipopolysaccharide (LPS) but with a shorter O antigen

Lipopeptides molecules consisting of a lipid covalently connected to a peptide; bacterial components recognized by TLR1

Lipopolysaccharide (LPS) a component of the gram-negative outer membrane; consists of lipid A (the toxic portion), a core made up of a series of sugars, and the O antigen, a long carbohydrate chain

Liposomes see **microspheres**

Lipoteichoic acid (LTA) a major component of the gram-positive bacterial cell surface consisting of a lipid-linked teichoic acid; the lipid is embedded in the cytoplasmic membrane, and the teichoic acid is exposed on the bacterial surface

Listeriolysin O (LLO) a pore-forming cytotoxin produced by *Listeria monocytogenes;* similar to enzymes produced by streptococci (streptolysin O and pneumolysin) and clostridia (perfringolysin O)

LLO see **listeriolysin O**

Locus of enterocyte effacement (LEE) a 35.6-kb pathogenicity island in the chromosome of EPEC that contains all EPEC genes necessary for the EAE phenotype

Loose connective tissue regions located under layers of epithelial cells; consists of extracellular matrix containing fibrous proteins, blood vessels, and other components

LOS see **lipooligosaccharide**

LPS see **lipopolysaccharide;** endotoxin

LPS O antigen see **O antigen**

LPS-binding protein (LBP) a plasma protein that binds lipopolysaccharide (LPS) and complexes with CD14 and TLRs on monocytes and macrophages to stimulate cytokine production through the TLR system

LPXTG an amino acid sequence motif of proteins cleaved by sortase of gram-positive bacteria and used to covalently link the cleaved protein to the peptidoglycan

LTA see **lipoteichoic acid**

***luc* gene** the gene for firefly luciferase

Luciferase (see also bioluminescence) an enzyme commonly used as a reporter of transcription; catalyzes the production of bioluminescence (light) through the oxidation of a pigment (luciferin)

LukF a component subunit of the leukocidin produced by *Staphylococcus aureus*

LukS a component subunit of the leukocidin produced by *Staphylococcus aureus*

Lumen the cavity in an organ not inside the body

lux the gene for bacterial luciferase

Lux see **bacterial luciferase and bioluminescence**

Lyme disease a systemic disease caused by *Borrelia burgdorferi* that is transmitted to humans by deer ticks

Lymph the fluid moving through the lymphatic system of the body; monitored by phagocytes

Lymph node an accumulation of lymphoid tissue positioned along lymphatic vessels that contain most of the lymphocytes of peripheral blood; the site where bacteria and toxins are removed from the circulation

Lymphadenopathy swollen lymph nodes; a diagnostic sign of infection

Lymphatic system a network of vessels that collects tissue fluid (lymph) and returns it to circulation; prevents excess buildup of fluid in tissues

Lymphocyte a leukocyte involved in immune response and chronic inflammation

Lymphokine a cytokine produced by lymphocytes

Lyophilization (freeze-drying) a procedure used for freeze-drying a sample that is carried out in a vacuum

Lysis disruption of a cell membrane to release its cytoplasmic contents

Lysogenic corynebacteriophages (β-phage and w-phage) lysogenic phages that carry genes for diphtheria toxin

Lysogenic phages (also lysogenic bacteriophage, temperate phage or **temperate bacteriophage)** bacteriophages whose genomes can integrate into the bacterial chromosome (lysogeny) or can excise from the chromosome and enter the lytic cycle in response to stress and kill the bacteria; may encode toxin genes (e.g., diphtheria toxin)

Lysogenic phase the phase in a bacteriophage reproductive cycle in which the bacteriophage genome is integrated into and maintained stably in the bacterial genome

Lysosomal granules see **lysosome**

Lysosome a mammalian cell organelle containing hydrolytic enzymes and other compounds toxic to bacteria

Lysozyme an enzyme that degrades glycan chains of bacterial peptidoglycan

LytA (also N-acetylmuramoyl-L-alanine amidase, cell wall hydrolase) an autolysin produced by Streptococcus during stationary phase that hydrolyzes the cell wall to cause autolysis

Lytic (i) see **lytic phase**; (ii) having the property of causing cell lysis

Lytic phase the stage in a bacteriophage reproductive cycle in which the bacteriophage replicates and lyses the bacterial host

M (microfold) cells cells in Peyer's patches that engulf bacteria from the lumen of the gut and deliver them to antigen-presenting cells (macrophages) in the gastrointestinal mucosa

M protein a *Streptococcus pyogenes* surface protein that binds to extracellular matrix proteins and the Fc portion of IgG; plays an antiphagocytic role

MAC see **membrane attack complex**

Macrolactone a small polyketide-derived lipid-like bacterial toxin

Macrolides a family of antibiotics that inhibit protein synthesis by binding to 23S rRNA in the 50S ribosomal subunit

Macrophage a large mononuclear antigen-presenting cell of the immune system having phagocytic activity; develops from activation of a monocyte during an immune response

Magnetotaxis the movement of bacteria toward a magnetic field

Major histocompatibility complex (MHC) a protein complex on the surfaces of macrophages that binds foreign (e.g., bacterial) peptides and displays them on the macrophage surface where they are recognized by the cognate receptors on T cells; there are two classes of MHCs: MHC I and MHC II

Malaise not feeling well; a general feeling of discomfort or uneasiness

MALT see **mucosa-associated lymphoid tissue**

Manhattan plot a type of scatter plot used to display data with large numbers of data points; commonly used in genome-wide association studies (GWAS) to display significant single nucleotide polymorphisms associated with risk of disease

Mannose-binding lectin (MBL) a calcium-dependent serum protein of the collectin protein family that is produced by the liver, binds to mannose found on bacterial surfaces, and activates complement

Margination the flattening of PMNs against a blood vessel wall prior to transmigration

Mass spectrometry (MS) an analytical technique that measures the masses of molecules within a sample by ionizing the molecules and separating the ions into a spectrum based on their mass-to-charge ratio; used to identify components in samples

Mast cells resident cells of several tissue types that contain granules rich in histamine, heparin, and other substances that when released can attract phagocytes to the site of bacterial invasion; also can produce cytokines

Matrix an artificial substrate used for binding and growing tissue culture cells

MBC see **minimal bactericidal concentration**

MBL see **mannose-binding lectin**

MBL-associated serine protease (MASP) (also mannose-binding lectin-associated serine protease) a serine protease enzyme involved the lectin pathway of the complement cascade that is responsible for cleaving complement components C4 and C2 into fragments that form the C3 convertase complex

MD2 an accessory protein for TLR4

MDR see **multidrug resistant**

***mecA* gene** the gene encoding a penicillin-binding protein (PBP2a) in *Staphylococcus aureus* that is not readily inhibited by methicillin

Mechanism-based inhibitor (suicide substrate) a compound that irreversibly blocks the active site of an enzyme

Mechanisms of antimicrobial resistance strategies used by bacteria to overcome the presence of antibiotics, including inactivation of the antibiotic, alteration of the antibiotic target, changes in membrane permeability, and efflux of the antibiotic out of the bacterial cell

Membrane attack complex (MAC) a multimeric complex of complement components C5b to C9 that binds to LPS on the cell surfaces of gram-negative bacteria and inserts into the membranes to form pores, thereby killing the bacteria

Membrane immunoglobulin (mIg) integral membrane protein on the surface of B cells that associates with other membrane proteins Igα and Igβ to form the B-cell antigen receptor complex (BCR)

Memory B cells see **memory cells**

Memory cells T or B cells or their progeny that persist for long periods in the body; they allow the body to respond rapidly to a second encounter with a microbe

Memory T cells see **memory cells**

Meninges the system of membranes covering the brain and spinal cord

Meningitis inflammation of the meninges

Metabolite-sensing riboswitches RNA elements that act as switches to control expression of genes involved in the biosynthesis of small ligands that bind to the switch; present in untranslated regions of the transcripts that they regulate

Metabolome the complete set and composition of all small molecule metabolites with a biological sample, comprised of a cell, tissue, organism, microbial community, or microbiome

Metabolomics the systematic study of the identification, quantification, and composition of small molecule metabolites (metabolome) of a biological system at a specific point of time; a temporal or spatial profile of the metabolome of a biological system

Metagenome all of the genetic material present in a sample; consists of the genomes of many different organisms

Metagenomic analysis a means of determining the genetic, proteomic, and metabolic potential of the microbiota

Metatranscriptomics the gene expression profiling of complex microbial communities within natural environments

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains of *Staphylococcus aureus* resistant to methicillin (as well as other antibiotics); currently treated with vancomycin

Metronidazole a synthetic antibiotic that makes breaks in DNA; must be activated by bacterial cell flavodoxin not present in host cells

MF59 a squalene adjuvant

MHC see **major histocompatibility complex**

MHC I an MHC type that when complexed with an epitope triggers activation and proliferation of $CD8^+$ cytotoxic T cells; found on most cells in the body

MHC I-specific inhibitory receptor surface receptors on natural killer (NK) cells that detect the loss of self-major histocompatibility complex (MHC) class I receptors (self-ligands) on the surface of host cells, which are often lost due to infection or other damage; receptors that mediate the missing-self response of NK cells

MHC II an MHC type that when complexed with an epitope leads to activation and proliferation of helper T cells; found on only a few cell types of the immune system (e.g., B cells and APCs)

Miasma theory an early, now obsolete medical theory that suggested the cause of disease was a poisonous vapor (bad air), arising from decayed organic matter (miasmata)

MIC see **minimal inhibitory concentration**

MICA and MICB protein complexes displayed on the surfaces of infected human cells; stimulate gamma-delta (g-d) T cells

Microarray see **microarray technology**

Microarray technology a multiplex biotechnique used in molecular biology to assess gene expression. The technique involves binding thousands of DNA oligonucleotide segments of genes (such as specific sequences of each gene in a genome) to a chip (made of a chemical, glass, or silicon matrix). Labeled probes of cDNA or cRNA from a sample (such as that obtained from RNA isolated from bacteria grown under different conditions) are then hybridized with the DNA on the chip to determine the relative abundances of the different target genes

Microbial community a group of microbes that share a common environmental niche in which they live

Microbial dysbiosis see **dysbiosis**

Microbial population shift see **dysbiosis**

Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) surface adhesins of *Staphylococcus aureus* and *Streptococcus* species that bind host extracellular matrix proteins

Microbicidal any agent that can be used to reduce the infectivity of a microbe by killing it

Microbiome the entire microbial populations, especially their genomes, in the human body and their interactions with their environment

Microbiostatic any agent that can be used to inhibit the growth or replication of a microbe

Microbiota the microbial communities of the body

Microbiota-gut-brain axis a biological systems model that proposes an important role for microbes in influencing the bidirectional communication and interactions between diet, gastrointestinal nutrition, metabolic and hormonal activity, gut and systemic immune function, cognitive function, neurodevelopment, and behavior

Microbiota shift disease a shift in the population of microbes that normally resides in a particular site, resulting in disease at that site

Microcapsules see **microspheres**

Microcin E492 a channel/pore-forming bacteriocin produced by *Klebsiella pneumoniae*

Microspheres beads made of resorbable inert material in which vaccine proteins are encapsulated; they serve as a type of adjuvant

Microvilli finger-like projections on the apical surfaces of mucosal absorptive cells

mIg membrane immunoglobulin expressed on the surface of a resting B cell

Minimal bactericidal concentration (MBC) the lowest concentration of antibiotic that will kill bacteria

Minimal inhibitory concentration (MIC) the lowest concentration of antibiotic that will prevent the growth of bacteria

Minor allele frequency the frequency at which the second most common allele or single nucleotide polymorphism (SNP) occurs in a given population

Mitochondria (singular mitochondrion) double-membrane organelle found in eukaryotic cells, in which the biochemical processes of respiration and energy production occur; phylogenetic evidence suggests that it derived evolutionarily from a bacterial endosymbiont

MLST see **multilocus sequence typing**

MMR vaccine a trivalent antiviral vaccine against measles, mumps, and rubella (German measles)

***mob* genes** genes on mobilizable plasmids that permit them to take advantage of transfer machinery of other, self-transmissible plasmids in the same cell

Mobile genetic element (MGE, also transposable elements) DNA segments that promote intracellular DNA mobility (movement within a genome) or that promote transfer from one species or replicon to another; types of MGEs are transposons, plasmids, bacteriophage, and self-splicing ribozymes

Mobilizable see **mobilizable plasmid**

Mobilizable plasmid a plasmid that is not self-transmissible but can be cotransferred with a self-transmissible plasmid

MOI see **multiplicity of infection**

Monobactams a class of antibiotics in the β-lactam family

Monocyte a mononuclear phagocyte circulating in blood; differentiates into a macrophage upon activation

Monophosphoryl lipid A (MPL) a TLR4-specific agonist; used as a vaccine adjuvant

Morbidity sickness

Moribund in a dying state; near death

Mortality fatality; lethality

MPL see **monophosphoryl lipid A**

MRSA see **methicillin-resistant *Staphylococcus aureus***

MSCRAMMs see **microbial surface components recognizing adhesive matrix molecules**

Mucin the high-molecular-weight, heavily glycosylated protein component of mucus; a complex, viscous, sticky mixture of glycoproteins covering mucosal membranes; produced and secreted by goblet cells onto mucosal surfaces

Mucoid a wet, glistening appearance of colonies of bacteria that produce capsules on agar plates

Mucosa-associated lymphoid tissue (MALT) the specialized immune system that protects all mucosal surfaces; includes GALT, BALT, SALT, and NALT

Mucosal epithelia the epithelial layer that lines respiratory, urogenital, and intestinal tracts; covered with a layer of mucus

Mucus see **mucin**

Multidrug efflux pumps integral membrane protein transporters that use energy to actively pump or excrete antibiotics and other small molecules out of the cell

Multidrug efflux system see **multidrug efflux pumps**

Multidrug resistant (MDR) resistant to more than one class of antibiotic

Multidrug-resistant bacteria strains of bacteria that are resistant to at least two different classes of antibiotic

Multidrug-resistant TB tuberculosis (TB) caused by strains of *Mycobacterium tuberculosis* that are resistant to most antibiotics commonly used against TB

Multifunctional autoprocessing repeats-in-toxin (MARTX) toxin a group of large, modular protein toxins found in *Vibrio* and other Gram-negative bacteria that are comprised of conserved repeated regions, an autoprocessing protease domain, and multiple intracellular toxic effector domains that get delivered into mammalian cells

Multilocus sequence analysis (MLSA) see **multilocus sequence typing**

Multilocus trait analysis (MLTA) see **genome-wide association studies**

Multilocus sequence typing (MLST) a multiplex method for sequencing DNA regions of several (seven or more) housekeeping or virulence genes at a time; allows comparison of different isolates of a single bacterial species

Multiomics a systems analysis approach that uses multiple types of datasets, such as genome, proteome, transcriptome, metabolome, and others (multi-omes) to study the biological system in a holistic and integrative way

Multiorgan microphysiological systems tissue-engineered, cell-based multiorgan models used for infectious disease studies and drug testing

Multiple organ system failure the cause of fatality in septic shock

Multiplexed (also multiplexing) an analysis method by which multiple signals, sensors, or other readouts are incorporated into a single platform for high-throughput assaying

Multiplicity of infection (MOI) a unit of measure used in an inoculation, defined as the ratio of infectious agent to target cell

Mupirocin an antibiotic effective against most MRSA strains that targets tRNA synthetases; cannot be used systemically because of rapid degradation but is used to clear nasal colonization by MRSA

Mycolactone a polyketide-derived lipid-like toxin produced by *Mycobacterium ulcerans* that has both cytotoxic and immunosuppressive properties; the causative agent of Buruli ulcers

Myeloperoxidase a lysosomal heme-containing peroxidase enzyme that forms reactive oxygen species from reaction of hydrogen peroxide with halides (such as chloride ion to form hypochlorous acid), which is highly toxic to bacteria during a respiratory burst

N-acyl homoserine lactones (AHLs) a class of intraspecies quorum-sensing signals used by gram-negative bacteria

NADPH oxidase a membrane-bound enzyme located in phagosomes that produces reactive oxygen intermediates when the phagosome fuses with the lysosome containing myeloperoxidase

Nalidixic acid a member of the quinolone family of antibiotics that inhibits DNA gyrase

NALT see **nasopharyngeal-associated lymphoid tissue**

Nanopore sequencing technology see **DNA sequencing technologies**

Nasal inoculation introduction of a vaccine by inhalation; stimulates the NALT

Nasopharyngeal-associated lymphoid tissue (NALT) the MALT of nasal passages

National Institutes of Health (NIH) a federal research and granting agency of the United States Department of Health and Human Services that is the largest biomedical research enterprise in the world with the goal of preventing disease, improving health and quality and usefulness of scientific information

National Microbial Pathogen Database Resource (**NMPDR**) a curated database of annotated genome data for a number of bacterial pathogens

National Science Advisory Board for Biosecurity (NSABB) a federal advisory committee comprised of leading experts that addresses issues related to biosecurity and dual-use life-science research and provides policy recommendations to US government agencies

Native PAGE a gel electrophoresis method that is non-denaturing such that mobility of a protein through the gel matrix depends on both the protein's charge and its hydrodynamic size

Natural killer (NK) cell a cytotoxic lymphocyte of innate immune defense cells that attacks infected host cells expressing antigen-bound MHC I molecules on their surfaces

Natural products antibiotics obtained from bacteria, fungi, plants, or animals isolated from soil or other environmental sources; usually products of secondary metabolism

Natural transformation the process by which DNA released from a donor cell into the environment is taken up by a recipient cell

Naturally competent see **naturally transformable**

Naturally transformable cells that are able to take up DNA from the environment without chemical treatment ("assistance")

NDP52 see **autophagy-related proteins**

Necrosis death of tissue; characterized by chromatin flocculation and disappearance of organelles

Necrotizing fasciitis a destructive wound infection caused by *Streptococcus pyogenes;* same as streptococcal gangrene

Neonatal-meningitis-causing *E. coli* (NMEC) strains of *Escherichia coli* that cross the intestinal mucosa of newborns and enter the bloodstream and then the cerebral spinal fluid to cause meningitis

Neuraminidase a glycoside hydrolase enzyme that cleaves the glycoside linkages of neuraminic acids

Neurotoxin a toxin specific for nerve cells

Neutralization of microbe or toxin blocking the action of a microbe or toxin by binding of specific antibodies to the surface of the microbe or to the toxin

Neutralization of the pathogen see **neutralization of microbe or toxin**

Neutropenia (also Neutropenic) a decrease in the number of neutrophils (PMNs) in the blood; immunodeficiency in neutrophils

Neutrophil also called polymorphonuclear leukocytes (PMNs); a leukocyte containing granules in which the granules do not stain dark using hematoxylin and eosin stains; one of the first inflammatory cells to migrate to the site of infection

Neutrophil extracellular traps (NETs) networks of extracellular fibers composed of DNA and degradative proteins released from neutrophils during infection, which bind pathogens and serve as a physical barrier that prevents microbial spread in the body and neutralize bacterial exoenzymes by proteolytic cleavage

Next-generation sequencing see **DNA sequencing technologies**

Nitric oxide (NO) a reactive nitrogen intermediate found in phagocytes; toxic to microbes; used as a mammalian signaling molecule

Nitrofurantoin a DNA-damaging antibiotic commonly used to treat urinary tract infections; requires activation by bacterial flavoproteins

NK cell see **natural killer cell**

NMEC see **neonatal-meningitis-causing *E. coli***

NO see **nitric oxide**

NOD1 and NOD2 intracellular pattern recognition proteins that recognize peptidoglycan inside host cells; analogous to TLRs

NOD17 see **NOD1 and NOD2**

NOD-like receptors (NLRs) intracellular pattern recognition receptor that recognize bacterial cell wall peptidoglycan motifs of N-acetylglucosamine and N-acetylmuramic acid crosslinked with peptide chains

Node in a phylogenetic tree that represents the evolutionary relationships among a set of organisms (or taxa), the tips represent the taxon (singular of taxa) and the nodes represent the last common ancestor of any two or more taxa

Nodosome intracellular protein complexes involved in pattern recognition receptors involving NOD proteins that sense intracellular pathogens and trigger inflammatory signals

Nonhomologous end joining (NHEJ) a pathway that repairs double-stranded DNA breaks by directly ligating the ends without using a homologous DNA strand as a template; not found in all bacteria

Nonoxidative killing a mechanism by which phagocytes kill bacteria in which toxic reactive oxygen species are not involved

Norfloxacin an antibiotic in the fluoroquinolone family

Normal microbiota see **resident microbiota**

Nosocomial infection (also hospital-acquired infection, hospital-associated infection, HA infection) an infection acquired in the hospital

NRAMP1 (natural resistance-associated macrophage protein-1) integral membrane metal ion (manganese or iron) transporter proteins; NRAMP1 is primarily expressed in macrophages and monocytes and prefers Mn^{2+} over Fe^{2+}

Nuclear localization signal (NLS) an amino acid sequence motif that tags a protein for import into the eukaryotic cell nucleus.

Nuclear magnetic resonance spectroscopy (NMR spectroscopy) an analytical technique used to determine the structure, properties and quantities of organic molecules in a sample by detecting the chemical shift of the resonance frequencies of the nuclear spins in the compounds; used to identify and quantify the molecules in a sample

Nucleoid a region of the prokaryotic cell that contains all or most of the genetic material

Nucleoid-associated proteins (NAPs) proteins found in the nucleoid of bacteria that help organize and compact the chromatin

Nutritional immunity process by which the host sequesters metal ions, such as iron, zinc and manganese, to limit pathogen access to them and reduce infection

O antigen the polysaccharide side chains on lipopolysaccharide (LPS) of gram-negative bacteria; used as the basis for determining the serogroup of a bacterial strain

O157:H7 the major pathogenic group of EHEC that causes bloody diarrhea and HUS

$O2^-$ see **superoxide radical**

Obligate required

Ocular infection an infection of the eye

ω-Phage a temperate bacteriophage that carries the gene for diphtheria toxin

$ONOO^-$ see **peroxynitrite**

Open reading frame (ORF) a segment of DNA sequence that encodes a putative polypeptide of unknown function; also see **URF**

Operational taxonomic unit (OTU) a term used to classify groups of closely related individuals based on sequence similarity; sequences are clustered according to their similarity to each other and the OTUs are defined based on the similarity threshold (often 97% similarity) set by the researcher

Operator a sequence of DNA to which a regulatory protein binds; usually used to denote the binding site of a repressor protein that blocks transcription

Operon a genetic unit of expression in bacteria consisting of a transcriptional start site; one or more genes, usually with related functions; and a transcriptional terminator

Operon fusion see **transcriptional fusion**

Opportunist see **opportunistic pathogen**

Opportunistic pathogen a microorganism capable of infecting and causing disease, but only when host defenses are compromised

Opsonin an antibody or complement component C3b that attaches to the bacterial surface and enhances the ability of phagocytes to engulf the bacterium

Opsonization the enhancement of phagocytosis (engulfment) by phagocytic immune cells by attachment of an antibody or complement component C3b to the bacteria

Opsonizing antibodies antibodies that attach to bacteria and enhance engulfment or opsonization by phagocytic cells

Optineurin (OPTN) see **autophagy-related proteins**

Oral rehydration therapy (ORT) a fluid rehydration therapy used to prevent and treat dehydration, especially due to diarrhea (such as cholera), comprised of an aqueous solution of salt, trisodium citrate, potassium chloride, and glucose

Oral vaccines vaccines administered by the mouth; they stimulate MALT

ORF see **open reading frame**

Organ cultures model systems comprising portions of organs kept viable in vitro; usually include multiple cell types

Oronasopharyngeal involving the mouth, nose and pharynx

Orthologs genes in different species evolved from a common ancestral gene

$OSCN^-$ see **hypothiocyanite**

Otitis media inflammation or infection of the middle ear

Outer membrane protein A (OmpA) a dominant integral membrane porin protein found in the outer membrane of *E. coli* and other Enterobacteriaceae

Oxazolidinones a class of antibiotics that bind to the 50S ribosomal subunit

Oxidative burst (respiratory burst) the production of reactive oxygen intermediates by phagocytes in the phagolysosomes

Oxidative killing the killing of bacteria by toxic reactive oxygen or nitrogen species inside phagocytes

P the whole-cell pertussis component of the DTP vaccine

p62 see **autophagy-related proteins**

Pacific BioSciences (PacBio) DNA sequencing see **DNA sequencing technologies**

PAF see platelet-activating factor

PAI see **pathogenicity island**

Paired-end sequencing (paired-end DNA sequencing) paired ends refers sequencing both ends of the same DNA fragment, which allows easier alignment and mapping of sequence reads because the distance between each paired end is known

PaLoc see **pathogenicity locus**

p-Aminobenzoic acid a substrate of the first enzyme in the tetrahydrofolic acid pathway; inhibited by the sulfonamide family of antibiotics

PAMPs see **pathogen-associated molecular patterns**

Pandemic an epidemic of an infectious disease that is spreading through many different countries or even worldwide

Pan-drug resistant (PDR) strains of bacteria that are resistant to multiple types of antibiotics; defined as nonsusceptibility to all drugs in all antibiotic categories

Paneth cells cells residing at that bottom of intestinal crypts that are key effectors of innate mucosal immunity by producing and secreting large amounts of α-defensins and other antimicrobial peptides, lysozyme, and phospholipase A2

Pangenome (also supragenome) a large gene pool comprised of the full complement of all core and distributed genes in a population of bacteria of the same species; because there can be large variation in gene content among closely related strains, this increases the opportunity for bacteria to easily acquire new genes

Panresistant see **multidrug resistant**

Panton-Valentine leucocidin (PVL) a β-pore-forming toxin from Staphylococcus aureus associated with increased virulence in certain clinical strains; comprised of LukS and LukF subunits

Paralogs gene copies created by a duplication event within the same genome

Parasite an organism that lives at the expense of another

Paroxysm a severe attack of symptoms (associated with coughing in whooping cough)

Partner-switching mechanism a regulatory signaling mechanism that occurs under stress conditions, whereby an anti-sigma factor forms alternative complexes with a sigma factor, keeping it inactive, or with an anti-anti-sigma factor, releasing the sigma factor so it can interact with RNA polymerase

Passive immunization the transfer of active humoral immunity by injecting antibodies against a particular pathogen or toxin obtained from an immunized person or animal into an unimmunized individual

***Pasteurella multocida* toxin (PMT)** a single-polypeptide AB-type toxin that interacts with and deamidates the α subunit of heterotrimeric G proteins (G_q or G_i), thereby disrupting host cell regulatory networks; causes cellular proliferation, bone destruction, and weight loss

Pasteurellosis a zoonotic disease caused by infection with *Pasteurella multocida*; the predominant diseases symptoms occur as upper respiratory flu-like pneumonia, septicemia, cellulitis, abscess formation, with dermonecrosis and bone loss in cases of bacteria that produce toxin

Pasteurization a semi-sterilizing method where food is treated with mild heat, usually less than 100°C (212°F) for a brief time

Passive immunity (also passive immunization) the transfer of exogenously made antibodies to a naïve individual to elicit active humoral immunity against an antigenic during an acute exposure or infection; maternal passive immunity is acquired by an infant from the mother through breastfeeding; artificial passive immunity is a short-term immunization acquired by transfer of antibodies via blood or serum from another person or an animal that has already been vaccinated

Patch a small piece of material containing many tiny prongs used to deliver vaccines via the skin

Pathogen a microorganism capable of colonizing a host and causing disease

Pathogen-associated molecular patterns (PAMPs) particular molecular features or motifs of bacteria and other pathogens that are recognized by special receptors of innate immune cells (TLRs and Nods) and play an important role in triggering innate immune responses

Pathogen evolution changes in genetic makeup that allow microbes to acquire new virulence factors or resist antibiotics

Pathogenicity the ability of a bacterium to cause disease

Pathogenicity island (PAI) a collection of genes involved in pathogenesis clustered together on DNA; usually acquired by horizontal gene transfer (HGT); often have a different G+C content than the rest of the bacterial chromosome

Pathogenicity locus (PaLoc) a pathogenicity island of *Clostridium difficile* containing genes encoding toxins A and B (TcdA and TcdB) and regulatory proteins

PBP2a a penicillin-binding protein; encoded by *mecA* in *Staphylococcus aureus*

PBPs see **penicillin-binding proteins**

PC see **phosphatidylcholine**

PCR see **polymerase chain reaction**

PCV-7 a seven-valent conjugate pneumococcal vaccine

PDB see **protein sequence database, protein structure database**

PDB RCSB Protein Data Bank, Research Collaboratory for Structural Bioinformatics; a protein structure model database

PE see **phosphatidylethanolamine**

PECAM see **platelet-endothelial cell adhesion molecule**

Pedestal formation raised actin structures formed by polymerization of host actin along with loss of nearby microvilli observed on intestinal epithelial layers, induced by toxic effector proteins secreted by extracellular pathogens, such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC); associated with the attaching and effacing phenotype

Penicillin an antibiotic in the β-lactam family

Penicillin-binding proteins (PBPs) proteins (normally located on the outer surface of the cytoplasmic membrane) that bind penicillins; include enzymes for peptidoglycan synthesis and turnover, especially transpeptidases

Peptidoglycan the polysaccharide backbone with peptide cross-links that covers the surface of the cytoplasmic membrane and gives bacteria their shape

Perforin a pore-forming cytolysin-like protein found in granules of CD8$^+$ cytotoxic T cells (CTLs) and natural killer (NK) cells; forms holes in membranes of host cells infected with intracellular pathogens

Periodontal disease (also Periodontitis) see **gingivitis**

Periodontium specialized tissue surrounding and supporting the teeth, including the gum, bone, cementum and periodontal ligament

Peroxiredoxin (**Prx**) a ubiquitous family of antioxidant enzymes that scavenge cellular reactive oxygen species and control cytokine-induced peroxide levels

Peroxynitrite (ONOO$^-$) a reactive nitrogen compound formed from reaction of nitrous oxide with superoxide radical; toxic for bacterial and human cells

Persister see **persister cells**

Persister cells a small percentage of nongrowing or dormant cells in a bacterial population that tolerates and survives antibiotic treatment and can resume growth once the antibiotic is removed, but still remains sensitive once regrown

Pertactin an adhesin on the surfaces of *Bordetella pertussis* cells

Pertussis (whooping cough) an acute respiratory disease caused by *Bordetella pertussis;* characterized by paroxysmal cough (whooping cough), excess mucus production, and vomiting

Pertussis toxin (PT, also PTx) a multisubunit AB-type protein toxin from *Bordetella pertussis;* consists of subunits S1 to S5, where S1 ADP-ribosylates G$_i$, a G protein that regulates host cell adenylyl cyclase activity, and the S2 to S5 subunits form a pentameric receptor-binding complex

Pertussis toxoid the inactive form of pertussis toxin used in the DTaP vaccine

Petechiae rash; skin lesions

Peyer's patches follicles (organized lymphoid tissue) in the small intestine that contain cells of the mucosa-associated immune system (MALT)

PFGE see **pulsed-field gel electrophoresis**

PG see **phosphatidylglycerol**

Phage see **bacteriophage**

Phage display regions from pathogen proteins incorporated into the coats of bacteriophage; used for screening potential vaccine targets

Phagocyte a host immune cell (white blood cell) adapted specifically to engulf (phagocytose) and destroy bacteria, dead or dying cells, and other foreign particulate matter

Phagocytic cell see **phagocyte**

Phagocytosis the ingestion (engulfment) of foreign particles by a phagocytic cell

Phagolysosome a vacuole resulting from the fusion of a phagosome and a lysosome

Phagosome a vacuole resulting from ingestion of particulate material, including bacteria, by phagocytes

Phalloidin a fungal toxin that binds tightly to F-actin but not G-actin; can be cross-linked to fluorescent dyes, such as rhodamine or FITC, for visualization of actin filaments in cells by fluorescence microscopy

Pharmacokinetics (PK) the study of the distribution of a compound (a drug, such as an antimicrobial compound) in the body

Pharyngitis inflammation of the pharynx; a sore throat

Phase variation a regulatory mechanism involving on-off control of some bacterial genes, such as those encoding surface proteins, that allow response to rapidly varying environmental conditions

Phenol-soluble modulin (PSM) a short amphipathic molecule; a weak toxin produced by *Staphylococcus epidermidis* and other *Staphylococcus* species

Phenotype a characteristic property or trait of an organism, such as morphology, growth rate, or ability to grow on selective media

PhoA see **alkaline phosphatase**

***phoA* gene** the gene encoding alkaline phosphatase; used as a reporter for expression of genes that are secreted

Phosphatidylcholine (PC) an abundant phospholipid found in biological membranes; structure consists of ethanolamine attached to the phosphate group of L-glycerol-3-phosphate backbone with ester bonds to saturated or unsaturated fatty acids in positions 1 and 2

Phosphatidylethanolamine (PE) an abundant positively charged aminophospholipid found in biological membranes

Phosphatidylglycerol (PG) a glycerophospholipid that is an integral part of the bacterial membrane; structure consists of L-glycerol-3-phosphate backbone with ester bonds to saturated or unsaturated fatty acids in positions 1 and 2

Phosphatidylinositol (PI) a negatively charged phospholipid found as a minor constituent of the cytosolic side of eukaryotic cell membranes that in eukaryotes may act as a second-messenger signaling molecule and as membrane anchor for carbohydrates and proteins on the outer cell surface membrane; structure consists of 1,2-diacyl-sn-glycerol-3-phospho-1'-myoinositol with ester bonds to saturated or unsaturated fatty acids in positions 1 and 2

Phosphatidylserine (PS) an abundant positively charged aminophospholipid found in biological membranes; structure consists of serine attached to the phosphate group of L-glycerol-3-phosphate backbone with ester bonds to saturated or unsaturated fatty acids in positions 1 and 2

Phospholipase an enzyme that hydrolyzes phospholipids that comprise the lipid bilayer of a host cell, thereby disrupting the cell membrane

Phosphorelay expansion of a two-component regulatory system to include a series of phosphotransferases that transfer the phosphoryl group from the sensor kinase to the final target response regulator

Phosphorylcholine (P-Cho) the polar head group of phosphatidylcholine (PC) and platelet-activating factor (PAF); structure consists of a negatively charged phosphate attached to a positively charged choline group

Phototaxis the movement of bacteria toward light

Phylochip a 16S rRNA gene microarray chip comprised of thousands of oligonucleotide spots, each corresponding to 16S rRNA genes from various microbial species in a sample; used for profiling microbial communities

Phylogenetic tree (also Phylogenetic relationship) a dendrogram or tree-like branching diagram that represents the evolutionary relationships among a group of organisms (or taxa) based on similarity to each other, where the tips represent each taxon (singular of taxa) and the nodes represent the last common ancestor of any two or more taxa

Phylotypes biological types or classification of organisms or taxa on the basis of their phylogenetic relationships

PI see **phosphatidylinositol**

Pili (also fimbriae) long, thick protein structures on the surfaces of bacteria that mediate adherence through a special protein(s) at the tip

Pilicide a new class of potential antibiotics designed to block pilus biogenesis

Pilin the major protein subunits packed in a helical array that form the shaft of a pilus

PK see **pharmacokinetics**

Plaque (i) the clear area formed in a monolayer of tissue culture cells when some of the cells are lysed by infecting bacteria, such as the clear area formed on fibroblast monolayers by *Listeria*; (ii) the clear area formed by lytic bacteriophage on an agar plate confluent with bacterial culture; (iii) a microbial biofilm formed on the surfaces of teeth (also called **dental plague**)

Plaque assay a technique using tissue culture cells to assess cell-to-cell spread of intracellular bacteria

Plasma the noncellular portion of blood that contains proteins, sugars, hormones, and elements necessary for clot formation

Plasma cell a mature form of a B cell that produces massive amounts of antibodies

Plasmid an autonomously replicating extrachromosomal DNA segment; most are circular, but *Borrelia* and a few other bacterial species have linear (self-complementary) plasmids

Plasmin a serine protease in blood that is involved in dissolving blood clots by degrading many blood plasma proteins, including fibrin clots (fibrinolysis)

Plasminogen the inactive precursor (zymogen) of plasmin

Platelet-activating factor (PAF) a cytokine produced by macrophages that contributes to dilation of blood vessels

Platelet activating factor receptor (PAFR) a G-protein-coupled receptor that binds platelet-activating factor (PAF); regulates signaling pathways involved in vasodilation, angiogenesis and inflammation

Platelet-endothelial cell adhesion molecule (PECAM) a protein that assists PMNs to move between endothelial cells

PLO see **pneumolysin**

PMN see **polymorphonuclear leukocyte**

PMT see ***Pasteurella multocida* toxin**

Pneumocin a bacteriocin produced by *Streptococcus pneumoniae* involved in intrastrain competition (bacterial interference) during colonization; small antimicrobial peptide that disrupt cell wall formation or membrane integrity

Pneumococcal adherence and virulence factor A (PavA) a surface protein of *Streptococcus pneumoniae* involved adhesion and invasion of lung cells

Pneumococcal surface adhesin (PsaA) an ABC-transporter lipoprotein complex on the surface of *Streptococcus pneumoniae* involved in manganese ion transport and adhesion; important for nasopharyngeal colonization

Pneumolysin (PLO) a cytotoxic, pore-forming protein produced by *Streptococcus pneumoniae;* similar to SLO, PFO, and LLO; binds to cholesterol in host cell membranes

PNSG see **poly-*N*-succinyl-β-1,6 glucosamine**

Polar a condition where the two ends of a structure differ from each other; for example, genetic elements or cells may be polar

Polarized cells mammalian cells that have different surfaces and different components on the different faces of the cell; most mucosal epithelial cells are polar

Poly-γ-glutamic acid (PGA) a polymer of repeating D- and L- glutamic acid residues, where the peptide bonds are formed between the amino group of on glutamic acid residue and the carboxyl group of the side chain of another glutamic acid residue; found in many *Bacillus* strains

Poly(I:C) double-stranded RNA made of I and C nucleotides; a synthetic double-stranded RNA that acts as a mucosal adjuvant

Poly-Ig receptor a receptor on the basal surface of mucosal epithelial cells to which IgA binds; responsible for transcytosis of IgA, a portion of which then becomes the secretory piece of sIgA

Polyketide synthases a family of large enzyme complexes that produce polyketides, secondary metabolites that are precursors to mycolactones, some antibiotics, and other toxins

Polymerase chain reaction (PCR) a method for amplifying DNA in vitro; involves priming with oligonucleotide primers complementary to nucleotide sequences flanking a target sequence with subsequent replication of the target sequence using a thermostable DNA polymerase

Polymorphonuclear leukocyte (PMN) a short-lived professional phagocyte (white blood cell) that circulates in the body; characterized by the presence of granules in the cytoplasm and varying shapes of the nucleus

Poly-*N*-succinyl-β-1,6 glucosamine (PNSG) a polysaccharide adhesin on the surfaces of *Staphylococcus aureus* and *Staphylococcus epidermidis* cells; adheres to plastic

Polysaccharide capsule one type of capsule made of exopolysaccharide molecules; imparts antiphagocytic properties to a pathogen

Polytopic membrane proteins integral transmembrane proteins where the polypeptide chain crosses the membrane multiple times

Polyvalent antigen an antigen with multiple antibody-binding sites

Pore-forming cytotoxin see **pore-forming toxin**

Pore-forming toxin a type of bacterial toxin that inserts into cell membranes to form pores and destroys the integrity of cell membranes

Porin a protein constituent of pores in the outer membrane of gram-negative bacteria that allows diffusion of nutrients

Positive feedback loop a process that occurs during a feedback loop that results in an effect, response or product that in turn positively affects or enhances the initial process to produce more of the effect, response or product; **A** makes more of **B** which in turn causes **A** to make even more of **B**

Postsurgical infection an infection that occurs after surgery; can cause serious complications

(p)ppGpp a bacterial compound that acts as a signal to change transcriptional patterns in response to amino acid limitation and other stresses; part of the stringent response system in bacteria

Prebiotics compounds that are supposed to foster growth of "good" bacteria in the gut

Precision medicine (also called personalized medicine) an approach to patient care where the doctor selects treatments that are tailored to the individual patient's unique medical history and genetic makeup, and a comprehensive understanding of their disease

Principal component analysis (PCA) a statistical method that uses orthogonal transformation to convert a large dataset of complex variables (multivariate dataset) into a smaller dataset (coordinate plot) that can reveal certain correlations or factors that contribute to the observed variance; used to visualize genetic distance and relatedness between samples or populations

***prfA* gene** a gene that encodes a positive regulatory protein that activates transcription of itself, listeriolysin O (LLO), and a number of other virulence genes in *Listeria monocytogenes*

Probiotic a preparation of live bacteria that is taken intentionally to bolster or alter the microbiota of the intestine or vagina

Progenitor toxin a toxin complex containing botulinum neurotoxin and other chaperone-like proteins that protect the neurotoxin from digestion in the stomach

Programmed cell death see **apoptosis**

Proinflammatory cytokines cytokines that aid in the process of inflammation

Promoter a site on DNA where RNA polymerase binds and initiates transcription

Properdin (factor P) a complement protein found in blood that positively regulates complement activation by binding to C3bBb complex and stabilizing the alternative pathway C3 and C5 convertases

Prophage a lysogenic bacteriophage that has integrated into the bacterial chromosome

Prophylaxis an action taken for protection against disease, such as giving antibiotics to people exposed to certain bacterial pathogens

Prospective study a study of disease or treatment in humans that begins in the present and moves into the future

Proteases enzymes that degrade proteins by hydrolyzing the peptide bonds

Protein A a surface protein of *Staphylococcus aureus* that binds the Fc portion of antibodies

Protein activity-modifying sRNAs a small group of small RNAs (sRNAs) that function by altering the activities of target proteins to which they bind

Protein-conducting channel an oligomeric assembly of SecYEG complexes through which secreted proteins are initially inserted

Protein microarray an orderly arrangement of proteins corresponding to all the genes in a genome that are embedded or covalently attached to a matrix, such as a membrane or chip

Protein database **see protein structure database**

Protein sequence database a bioinformatics resource containing a collection of amino acid sequences of specific proteins; sequence databases can be searched using a variety of search programs, such as BLAST

Protein structure database (also protein data bank, PDB) a bioinformatics resource containing a collection of 3-dimensional structures of specific proteins and nucleic acids

Proteoarray see **protein microarray**

Proteomic profiling a high-throughput microarray technique for determining patterns of gene and protein expression in bacterial or host cells; can be used as a diagnostic tool

Proteomics see **proteomic profiling**

Proton motive force the energy that is generated by the transfer of protons or electrons across an energy-transducing membrane and that can be used for chemical, osmotic or mechanical work; the force that promotes movement of protons across membranes down an electrochemical potential

Protospacer-adjacent motif (PAM) a DNA sequence made of 2-6 base pairs immediately downstream of the DNA sequence (in the invading virus or plasmid) targeted by the Cas9 nuclease in the bacterial CRISPR-Cas system

Protozoa single-celled eukaryotes

PS see **phosphatidylserine**

Pseudogenes genomic DNA sequences that are related to known genes, but that have lost at least some functionality in either expression of the gene or ability to express functional protein; defunct relatives of functional genes

Pseudomembrane a sheet-like layer of debris (fibrin, mucin, and dead host cells) that covers a large area of the colon (pseudomembranous colitis) or the throat (diphtheria)

Pseudomembranous colitis infection of the colon characterized by a pseudomembrane

PSM see **phenol-soluble modulin**

Psoralen a furocoumarin compound that upon UV irradiation cross-links DNA, thereby blocking DNA synthesis, replication, and repair in bacteria, but not protein synthesis or metabolism; used to produce live vaccines with bacteria that do not proliferate but do produce antigens

PT see **pertussis toxin**

ptsAB genes required for secretion of pertussis toxin

PTx see **pertussis toxin**

ptxAB genes encoding pertussis toxin subunits

PubMed a bioinformatics database for life science literature sources

Pulldown assay see **immunoprecipitation**

Pulsed-field gel electrophoresis (PFGE) an electrophoretic separation technique in which large pieces of DNA are subjected to bursts of electrical charge in different directions while migrating through a gel; used to compare bacterial strains in disease outbreaks

Purulent associated with the formation of pus

Pus whitish to brownish exudates produced by vertebrates during an inflammatory response to infection involving the accumulation of fibrin, PMNs, fragments of host cells, and released DNA

Pyocyanin a blue redox-active, toxic, phenazine-derived, secondary metabolite produced by Pseudomonas aeruginosa; released in large amounts into the lungs of infected cystic fibrosis patients

Pyogenic pus forming

Pyrogenic fever inducing

Pyroptosis highly inflammatory form of programmed cell death mediated by caspase-1 signaling that can occur during infection with intracellular pathogens

Pyruvate oxidase (SpxB) an enzyme of *Streptococcus pneumoniae* and some other bacterial species that produces endogenous hydrogen peroxide

QACs see **quaternary ammonium compounds**

qPCR see **quantitative** real-time PCR

QS-21 a derivative of QuilA that is used as an adjuvant

Quantitative real-time PCR (qPCR) a PCR-based method for determining the relative numbers of different microbial species in a population; based on calculating the relative concentrations of 16S rRNA genes of each species; can be combined with reverse transcription to determine relative transcript amounts (RT-qPCR)

Quantitative trait a measurable phenotype (or characteristic) that is controlled by multiple genes and can be influenced by environmental factors

Quantitative trait loci (QTL) a region of genomic DNA that is associated or correlated with variation of a particular phenotypic trait, which varies in degree (can be quantified) and can be attributed to multiple genes and environmental factors; QTLs are often found on different chromosomes

Quaternary ammonium compounds (QACs) disinfectants that intercalate into phospholipid bilayer membranes causing bacteria to lose essential ions and other small molecules

QuilA a saponin vaccine adjuvant

Quinolones a family of antibiotics that inhibit DNA gyrase; includes nalidixic acid

Quinupristin a streptogramin antibiotic

Quorum the minimum number of a group of bacteria that act together to generate a particular response

Quorum-sensing system a regulatory system that recognizes a particular bacterial signal (autoinducer), thereby sensing the density of bacteria in the area; controls either repressors or activators of specific sets of genes in response to bacterial population density

Quorum sensing the ability of a bacterium to sense and quantify when other bacteria are present and then to generate a particular response

R domain the portion of diphtheria toxin that binds to a protein receptor on a host cell

Rabbit ileal loop model a procedure for testing the impacts of toxins and other diarrheal agents on the small intestine, whereby a segment of the rabbit intestinal ileum is isolated and the toxin, bacterium, or other potential diarrheal agent is injected into the lumen and after a period of time is measured for distension or swelling to indicate whether the agent has diarrhea-inducing properties

Rac (also Rac1 GTPase) a small G-protein (GTPase) related to Rho GTPases that is inactive when bound with GDP and active when bound with GTP and that regulates many eukaryotic cellular processes, including cell cycle, cell-cell adhesion, actin motility, and epithelial differentiation; a substrate of certain bacterial protein toxins, including ADP-ribosyltransferases, such as the *Clostridium botulinum* exoenzyme C3, and G-protein-deamidases, such as the cytotoxic necrotizing factors (CNFs) from *E. coli*

Rac 18 a protein targeted by toxins A and B of *Clostridium difficile*

RAG-1 (recombination-activating gene 1 protein, also RAG-2) proteins involved in activation of immunoglobulin V-D-J recombination; RAG1 binds to DNA substrate, while RAG2 binds and cleaves; the RAG-1/2 complex binds to recombination signal sequences that flank the V, D, J regions in a gene and nicks the DNA; deletion in RAG-1 are used in mouse models to impair maturation of T cells and B cells

Rational drug design an approach to drug development based on the synthesis of chemicals designed to bind to and inactivate a host target molecule based on the structures of the compound and target

RDB see **Ribosomal Database**

Recipient see **recipient cell**

Recipient cell a cell that takes up DNA (i) from the environment or (ii) directly from another cell

Red fluorescent protein (RFP) a small protein produced by *Discosoma* (called DsRed) that fluoresces red-orange when excited; used in molecular biology applications as a reporter gene for detection of gene expression or in cell biology applications for visualization of cellular proteins either as RFP fusion proteins or as fluorophores attached to antibodies that recognize cellular proteins

Reemerging infectious diseases the appearance of older diseases once thought to be under control

Regulator a protein that controls the expression or activity of another target protein (e.g., a protein that controls transcription of a specific gene or set of genes)

Regulon genes located at different locations that have promoter/operator or promoter/activator binding regions that all recognize the same regulatory protein(s)

***Renilla* luciferase** see **bioluminescence**

Reporter fusion see **transcriptional fusion**

Reporter gene a gene encoding an easily assayable enzyme or a readily observable fluorescent protein that is used in transcriptional fusions or translational fusions to measure gene expression

Reservoir a group of microorganisms that serve as a source for new genetic material

Resistome the collection of all antibiotic resistance genes found in pathogenic and nonpathogenic bacteria that serves as reservoir for conferring resistance

Resfams a curated antibiotic resistance gene database of protein families and associated profiles used for annotation of antibiotic resistance genes

ResFinder a bioinformatic application tool used for identifying horizontally acquired antibiotic resistance genes in bacteria

Resident microbiota a population of normally nonvirulent bacteria found routinely in a site in the bodies of most healthy adults or children

Respiratory burst see **oxidative burst**

Response regulator one component of a two-component regulatory system that mediates the cellular response; usually a DNA-binding protein that changes transcription in response to phosphorylation

Restriction enzyme (also restriction endonuclease, deoxyribonuclease, DNase) a prokaryotic site-specific DNase that recognizes specific sequences in DNA and cleaves the DNA at or near those sites

Restriction-modification system (RM system) a prokaryotic defense system that protects the bacteria or archaea from acquiring foreign DNA, such as plasmid or phage DNA; most commonly consists of a DNA methylase responsible for modification of the DNA and an endonuclease responsible for cleaving the unmodified DNA

Retrospective study a study of disease outbreaks after the fact that provides information on disease transmission or progression in humans by analyzing previously accumulated data

RGD tags Arg-Gly-Asp peptide motifs that act as ligands for cell surface integrins

Rhamnolipids a glycolipid produced by Pseudomonas aeruginosa that act as a surfactant; structure consists of a polar rhamnose sugar unit linked to a lipid moiety

Rheumatic fever a febrile illness that occurs several weeks after infection (sore throat) with *Streptococcus pyogenes*; can be accompanied by damage to heart valves

Rheumatic heart disease heart valve damage following rheumatic fever

Rho mammalian small G proteins (GTPases) targeted by a number of bacterial protein toxins, such as the cytotoxic necrotizing factors of *Escherichia coli* and toxins A and B of *Clostridium difficile*

Rhodamine a red (fluorone) fluorescent dye

Rho-dependent terminators proteins that mediate transcription termination by Rho (ρ) factor

Rho-dependent transcriptional terminators RNA structures that require bacterial Rho (r) factor protein to cause transcription termination

Rho GTPase a family of small G-proteins (RhoA, Rac1, Cdc42) involved in signal transduction; have GTPase activity and are inactive when bound with GDP and active when bound with GTP and that regulate many eukaryotic cellular processes, including cell cycle, cell-cell adhesion, actin motility, and epithelial differentiation; a substrate of certain bacterial protein toxins, including ADP-ribosyltransferases, such as the *Clostridium botulinum* exoenzyme C3, and G-protein-deamidases, such as the cytotoxic necrotizing factors (CNFs) from *E. coli*

Rho (ρ) factor a prokaryotic protein involved in termination of transcription; binds to the C-rich (G-poor) transcription terminator pause site (called *rut*) of single stranded RNA being synthesized upstream of the actual terminator sequence and moves along it in a 5’ to 3’ direction, causing the RNA polymerase to dissociate from the DNA template

Rho-independent termination (intrinsic termination) transcription termination mediated by the newly synthesized RNA forming a hairpin structure that displaces the RNA polymerase from the DNA template to stop transcription

Ribosome-binding site (RBS; also called Shine-Dalgarno [SD] sequence in bacteria) a sequence upstream of the start codon (most commonly AUG) of an mRNA transcript that is used to recruit the ribosome during initiation of protein translation

Ribosomal Database (RDB) a bioinformatics database that provides online data analysis, alignment, and annotation of bacterial and archaeal small-subunit 16S rRNA gene sequences

Ribosome protection protection of ribosomes from tetracycline by a bacterial cytoplasmic protein

Riboswitches a regulatory segment of mRNA that binds to a small molecule and modulates translation of the mRNA, thereby changing protein expression levels

Rice-water stool stool from patients suffering from cholera diarrhea, where the stool resembles water in which rice has been rinsed

Ricin an AB-type protein toxin produced by castor beans (*Ricinus communis*) that is comprised of a lectin (carbohydrate-binding protein) chain that binds galactose groups on cell surfaces and a ribosome-inactivating protein (RIP) chain with N-glycoside hydrolase activity that removes the adenine base at position 4324 within the 28S rRNA, leading to ribosome inactivation and inhibition of protein synthesis

Rifampin (also Rifampicin) an antibiotic that inhibits bacterial RNA polymerase

RNA interference (RNAi) the posttranscriptional silencing of specific genes by double-stranded RNA; involves two types of small RNA, micro-RNA (miRNA) and short-interfering RNA (siRNA), that bind to mRNA and induce the Dicer complex that degrades double-stranded RNA and prevents expression of proteins

RNAi see **RNA interference**

RNA-Sequencing (RNA-Seq) see **RNA-Seq technology**

RNA-Seq technology (also called whole transcriptome shotgun sequencing) transcriptomic profiling of the amounts of cellular RNA transcripts to determine the physiological state of the cell (bacterial or host) under a given condition; see also **dual RNA-Seq technology**

RuvC (i) resolvase (endonuclease) that cleaves the Holliday junction during homologous recombination and post-replication repair; (ii) nuclease domain of Cas9 that initiates cleavage of the DNA strand not complementary to the guide RNA in CRISPR-Cas systems

16S rRNA gene microarray chips see **phylochips**

SAg see **superantigen**

Sak see **staphylokinase**

Salmochelin an iron-chelating, enterobactin-like (catechol-like) siderophore produced under iron-poor conditions by *Salmonella enterica*, certain pathogenic *E. coli*, and certain *Klebsiella* strains

***Salmonella enterica* serovar Typhimurium DT104** a multidrug-resistant strain responsible for outbreaks among humans in Europe and the United States

SALT see **skin-associated lymphoid tissue**

Sanger sequencing a method of DNA sequencing based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication; most commonly used method for detection of single nucleotide polymorphisms (SNPs) or single nucleotide variants (SNVs)

Saponins plant-derived triterpene glycosides used as vaccine adjuvants

SAR see **structure-activity relationship**

Scalded-skin syndrome a disease in infants caused by exfoliative toxins produced by *Staphylococcus aureus* where the upper layers of skin peel off

SCC*mec* see **staphylococcal cassette chromosome, *mec***

SCID (severe combined immunodeficiency) a primary immune deficiency disease characterized by a severe immunocompromised state due to a defect in both T cell and B cell lymphocytes that causes severe susceptibility to microbial infections

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) an electrophoresis method for separating macromolecules, especially proteins, based on their electrophoretic mobility through a polyacrylamide-based gel matrix, using SDS to denature the proteins and enable differentiation based on molecular size

SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE different antigenic types of staphylococcal enterotoxins (SEs) produced by *Staphylococcus aureus;* SEA is the most common cause of foodborne disease

Sebaceous (fat) glands skin glands that produce and secrete sebum

Sebum an oily, waxy substance consisting of triglycerides, wax esters, squalene, and metabolites of fat-producing cells; waterproofs and lubricates the skin and hair of mammals

Sec system see **general secretory pathway**

SecA a molecular motor whose ATPase activity provides part of the energy for the protein translocation process of the general secretory pathway

SecA1 a protein that is part of the accessory Sec system; closely related to SecA proteins of other systems

SecA2 a protein that is part of the accessory Sec system

SecB a protein chaperone that binds to proteins targeted for secretion through the general secretory pathway

Secondary metabolites compounds that appear to have no direct role in energy metabolism or essential biosynthetic reactions; examples are pigments and antibiotics

Secretory piece a portion of the IgA receptor of mucosal cells that becomes attached to IgA as it passes through the mucosal cells; the secretory piece plus IgA becomes sIgA

SecYEG subunits of the protein-conducting channel of the general secretory pathway

Select agents a list of bacteria, viruses, and toxins that are considered to be potential bioterror agents (biothreats)

Selectins a family of cell adhesion molecules (CAMs); surface transmembrane glycoproteins of endothelial cells that bind to PMNs

Self antigen any molecule or substance of an organism that acts as an antigen to induce antibody production in another recipient organism, but that are not recognized as foreign by the donor organism; antigens in one's own body that differ from the analogous antigens from another person or animal and so are recognized as foreign antigens by the immune system of the other person or animal

Self-transmissible plasmid see **conjugative plasmid**

Semisynthetic antibiotics antibiotics originally isolated from nature but then chemically modified to improve their characteristics

Sensor kinase see **histidine kinase**

Sepsis (i) a condition resulting from microbes or microbial products in the blood; (ii) systemic inflammatory response syndrome plus culture-documented infection

Septic shock a systemic reaction caused when bacterial cell wall components (LPS, LTA, and peptidoglycan fragments) trigger the release of cytokines that have a variety of effects on control of body temperature and blood pressure; symptoms include fever, hypotension, disseminated intravascular coagulation, acute respiratory disease, and multiple organ system failure

Septicemia a systemic disease in which microorganisms multiply in the blood or are continuously seeded into the bloodstream

Sequencing technology see **DNA sequencing technologies**

Serogroup a classification of bacterial strains based on a particular group of bacteria containing a common, shared antigen, such as the O antigen of LPS for classification of *Escherichia coli* strains

Serological classification (serogroup; serotype) a scheme based on reactivity of bacterial surface antigens with antibodies (e.g., O antigen of LPS, H antigen of flagella, and C antigen of streptococci); see **Lancefield grouping system**

Serotonin (5-hydroxytryptamine) a neurotransmitter derived from tryptophan produced in the central nervous system and in the enteric nervous system located in the gastrointestinal tract, stored in blood platelets and released during agitation and vasoconstriction

Serotype the serological classification of a bacterial subgroup within a species (e.g., classification of *Escherichia coli* based on H antigen or of *Streptococcus pneumoniae* based on capsule antigens)

Serotyping a classification of bacteria based on the reactivities of their surface molecules with different antibodies

Serovar a subgroup classification of botulinum neurotoxins based on the non-cross-reactivity of antibodies against one group of the toxin proteins with those of another group

Serum the fluid portion of the blood without clotting factors; the component of blood that is collected after coagulation; contains antibodies

Serum resistant describes bacteria that can resist the killing action of serum; in gram-negative bacteria, serum resistance results from alteration in the O antigen so that MAC cannot nucleate around LPS

Serum sensitive describes bacteria that cannot resist the killing action of serum; gram-negative bacteria that are susceptible to MAC

SEs see **staphylococcal enterotoxins**

Severe sepsis the third stage of septic shock characterized by organ dysfunction and low blood pressure

Sexually transmitted disease (STD) disease transmitted by sexual activity

Shiga-like toxin (SLT) an AB-type exotoxin produced by some species of intestinal pathogens (e.g., EHEC); has an activity like that of Shiga toxin

Shiga toxin (STx) an AB-type protein toxin produced by *Shigella* that cleaves rRNA and stops protein synthesis in host cells; responsible for hemolytic uremic syndrome (HUS) during *Shigella*, EHEC or STEC infection

Shiga toxin (STx)-producing *E. coli* (STEC) foodborne *E. coli* strains (such as O157:H7) that cause hemorrhagic colitis and hemolytic uremic syndrome (HUS), primarily through the production of Shiga toxin variants

Shigella dysenteriae a gram-negative bacterium that causes dysentery; produces Shiga toxin

Short interfering RNA (siRNA) double-stranded RNA molecules 21 to 23 nucleotides long that bind to homologous mRNA and that induce the Dicer complex to cause selective degradation of that mRNA

Shotgun sequencing see **DNA sequencing technologies**

Sialic acid N- or O-substituted derivatives of neuraminic acid, a nine-carbon sugar found commonly on mammalian cell glycolipids and glycoproteins; used by some pathogens to coat their surfaces and evade the host immune response by mimicking host cell surfaces

Sialyltransferase enzymes that transfer sialic acid groups to nascent oligosaccharides, terminal sugars of glycolipids (gangliosides), or N- or O-linked sugar chains of glycoproteins

Siderophores low-molecular-weight compounds produced by bacteria that chelate iron; involved in iron acquisition by bacteria

sIgA (secretory immunoglobulin A, also IgA) see **immunoglobulin A**

sIgA protease a bacterial enzyme that cleaves human sIgA at the hinge region

Sigma factor a subunit of bacterial RNA polymerase that allows RNA polymerase to recognize a particular class of promoter; binds to the core RNA polymerase subunits to form a holoenzyme

Signal recognition particle (SRP, also called the docking protein) a ribonucleoprotein (protein-RNA) complex to which signal peptides of integral membrane proteins bind and are targeted to the endoplasmic reticulum in eukaryotes or the plasma membrane in prokaryotes

Signal-recognition particle (SRP) complex a cytosolic ribonucleoprotein (protein-RNA complex) that recognizes and targets specific proteins to the endoplasmic reticulum in eukaryotes and to the plasma membrane in prokaryotes

Signal sequence a sequence of 15 to 26 amino acids at the N termini of proteins that target them for secretion via the general secretory system

Signal transduction a cascade of signaling proteins that mediate messages within a cell through external stimulation of a bacterial or mammalian cell surface receptor, which triggers a set of protein modifications, such as phosphorylation/dephosphorylation, that affect gene expression and metabolism

Sigma (σ) factors a bacterial transcription initiation factor that enables specific binding of RNA polymerase to gene promoters

Simple epithelium a monolayer of epithelial cells covering body surfaces where absorption or secretion takes place

Single guide RNA (sgRNA) a single engineered RNA molecule that contains both the custom-designed short crRNA sequence fused to the scaffold tracrRNA sequence, used to direct the Cas9 protein to bind and cleave a particular DNA sequence in genome editing applications

Single nucleotide polymorphism (SNP) a spontaneous mutation in which single nucleotides are changed; can result in amino acid substitution or protein chain termination

Single-molecule, real-time (SMRT) sequencing see **DNA sequencing technologies**

siRNA see **short interfering RNA**

SIRS see **systemic inflammatory response syndrome**

Site-specific DNA resolvase a protein that causes cleavage of DNA at a specific recognition sequence

Site-specific recombinase see **integrase**

Skin-associated lymphoid tissue (SALT) a specialized set of cells that confront bacterial invaders in the area immediately underlying the skin and attempt to prevent their access to the bloodstream

Slipped-strand misrepair (also slipped-strand synthesis) a process by which phase variation can occur; results from a frameshift during DNA replication in regions of highly repetitive sequence; a frequent basis of phase variation in *Neisseria gonorrhoeae*

Slipped-strand synthesis see **slipped-strand misrepair**

SLO see **streptolysin O**

SLT see **shiga-like toxin**

Small MASP-associated protein (sMAP) a truncated form of MASP-2 that is also associated with MBL/ficolin-MASP complexes

Small Rho GTPase see **Rho GTPase**

Small RNA (sRNA) transcripts that are complementary to mRNAs of target genes; can modulate levels of virulence proteins

SNAP25 synaptosomal-associated protein 25; a SNARE complex protein associated with the plasma membrane in neurons that facilitates synaptic vesicle-plasma membrane fusion during neurotransmission; complexed with the synaptic vesicle protein synaptobrevin (also called VAMP) and another plasma membrane protein, syntaxin; serves as a substrate for cleavage by botulinum neurotoxins (BoNTs)

SNARE proteins a set of neuronal proteins (comprised of SNAP-25, synaptobrevin, and syntaxin) that form a SNARE complex involved in synaptic vesicle fusion with the plasma membrane during neurotransmission; intracellular targets of botulinum neurotoxins (BoNTs)

SNP see **single nucleotide polymorphism**

Sod see **superoxide dismutase**

Somatic hypermutation (i) adaptation of the immune system in response to new foreign elements (antigens from microbes), as occurs during class switching; (ii) high frequency of point mutations generated within the variable region of expressed immunoglobulin genes in response to the presence of antigen

Sortase a membrane transpeptidase of streptococci and other gram-positive bacteria that cleaves polypeptides between the threonine and glycine of the LPXTG motif and catalyzes covalent peptide bond formation between surface proteins, including pilin subunits, and amino acids in the peptidoglycan of the cell well, thereby anchoring the proteins to the bacterial cell surface

Specialized transducing phages lysogenic phages that can transduce bacterial sequences close to the phage attachment site, thereby packaging some of the host DNA along with the phage DNA in the phage particle; they undergo both lytic and lysogenic phases

Specific-pathogen-free (SPF) animals animals raised in an environment free of a particular pathogen or pathogens, yet naturally colonized by other microbes

SPF see **specific-pathogen-free animals**

Spleen macrophages resident (fixed) macrophages of the spleen

Spontaneous generation an earlier, obsolete theory of how living matter such as germs could arise from nonliving matter, used to explain how germs could appear in supposedly sterile environments

Spore a metabolically dormant form of some bacteria, derived from vegetative cells, that is highly resistant to extreme environmental conditions

Sputum the material coughed up from an infected lung

Squalene a precursor of cholesterol and other steroids; a component of a new adjuvant approved for use in humans

Squamous epithelium flattened scale-like epithelial cells on the outer layer of the skin and other surfaces, e.g., the stomach lining and respiratory and digestive tracts

sRNA see **small RNA**

SRP see **signal recognition particle**

SSH see **suppressive subtractive hybridization**

STx see **shiga toxin**

Staphylococcal cassette chromosome, *mec* (SCC*mec*) a mobile genetic element containing the *mecA* gene and the genes regulating its expression in *Staphylococcus aureus*

Staphylococcal enterotoxins (SEs) superantigens produced by *Staphylococcus aureus*

Staphyloferrin a siderophore produced by *Staphylococcus* species

Staphylokinase (Sak) an exoprotein toxin produced by *Staphylococcus aureus* that inactivates plasminogen, leading to dissolution of clots

Staphyloxanthin a carotenoid pigment produced by *Staphylococcus aureus* that is responsible for the characteristic golden color of the cells; acts as an antioxidant that scavenges reactive oxygen species and helps the bacteria survival oxidative burst

Stem-loop structure an internal paired structure that forms in mRNA that can be used in regulatory mechanisms, such as erythromycin resistance

Stochastic processes the random noise (chance events) in regulatory circuits that can lead to subpopulations with different phenotypes and environmental responses

Stratified epithelium multiple layers of cells that make up the epithelium

Strep throat pharyngitis due to *Streptococcus pyogenes*

Streptococcal gangrene see **necrotizing fasciitis**

Streptogramins a group of antibiotics that act by binding the ribosome and preventing protein translocation

Streptokinase a blood clot-dissolving enzyme produced by *Streptococcus pyogenes* that binds and activates plasminogen to produce plasmin

Streptolysin O (SLO) an oxygen-labile pore-forming toxin produced by *Streptococcus pyogenes*

Streptomyces a group of Gram-positive bacteria that produce many different antibiotics

Structure-activity relationship (SAR) a process used in drug discovery to modify chemicals and determine the effects of the changes on biological drug properties, such as antibiotic efficacy

Substrate see **matrix**

Subunit vaccine a vaccine that consists of a few purified proteins, such as detoxified toxins, adhesins, or other bacterial surface proteins

Suicide substrate see **mechanism-based inhibitor**

Sulbactam a suicide-substrate-based inhibitor of β-lactamase

Sulfonamides a family of antibiotics that inhibit an enzyme in the pathway that leads to synthesis of tetrahydrofolic acid; sulfa drug antibiotics

Superantigen (SAg) peptide toxins that bind to MCH-TCR complexes with or without specific antigen bound and thereby stimulate large populations of T cells to produce cytokines

Superbugs microorganisms that are resistant to most antibiotics

Supercoiling a topological state of closed, circular, double-stranded DNA where the DNA strands are momentarily broken, twisted around their axis, and rejoined

Superoxide dismutase (Sod) an enzyme that catalyzes conversion of superoxide radical to hydrogen peroxide

Superoxide radical (O2^{-}) a reactive oxygen species produced during phagocytosis

Suppurative producing pus, as in an abscess

Supragenome see **pangenome**

Surface adhesin a molecule found on the surface of bacteria that recognizes and binds to a host cell receptor molecule

Surveillance programs programs developed to monitor the appearance of new diseases, increased incidence of known diseases, antibiotic-resistant bacteria, and other emerging infectious disease or epidemiological patterns

Swiss-Prot Swiss Institute of Bioinformatics protein sequence database

SYBR green a dye that binds to double-stranded PCR products; enhances fluorescence

Symptoms the physiological effects of bacterial colonization or invasive disease that are apparent to the infected host

Synaptobrevin (also VAMP) a SNARE complex protein associated with the synaptic vesicle membrane in neurons that facilitates synaptic-vesicle–plasma membrane fusion during neurotransmission; complexed with the plasma membrane proteins SNAP-25 and syntaxin; serves as a substrate for cleavage by botulinum neurotoxins (BoNTs)

Syndrome the symptoms that characterize a specific disease

Synercid an antibiotic consisting of two streptogramin antibiotics

Syntaxin a SNARE complex protein associated with the plasma membrane in neurons that facilitates synaptic-vesicle–plasma membrane fusion during neurotransmission; complexed with the synaptic vesicle protein syntaxin and another plasma membrane protein, SNAP-25; serves as a substrate for cleavage by botulinum neurotoxins (BoNTs)

Syphilis a multi-stage, sexually transmitted disease caused by the spirochete *Treponema pallidum*

Systemic affecting the whole organism rather than a specific organ or tissue

Systemic inflammatory response syndrome (SIRS) the first stage of septic shock

Systems genetics an integrative multiomics approach that uses a wide range of experimental and statistical tools to quantitate and integrate phenotypes with genotypes in populations that vary for certain traits of interest so as to understand the flow of biological information that contributes to complex traits

T the tetanus toxoid component of the DTP or DTaP vaccine

T4SS-mediated DNA conjugation transfer of DNA (conjugation) through a type 4 secretion system (T4SS) from a donor cell to a recipient cell

T cell a thymus-derived lymphocyte of the adaptive immune system; T helper cells activate macrophages (Th1) or stimulate antibody production by B cells (Th2); cytotoxic T cells (CTLs) kill host cells infected by specific intracellular pathogens

T domain membrane translocation domain found in many single polypeptide AB-type toxins, such as diphtheria toxin

T helper (Th) cells T cells that activate macrophages and other immune cells or stimulate antibody production by B cells by releasing T cell cytokines; they have CD4 on their surfaces; Th0 cells differentiate into Th1 cells that stimulate cell-mediated immune responses, into Th2 cells that stimulate humoral immune responses, into Th17 cells that produce IL-17, or into Treg cells that suppress immune responses

TA teichoic acid

TALEN (transcription activator-like effector nuclease) restriction nuclease enzymes that can be engineered to cleave specific sequences in DNA, made by fusing a TAL effector DNA-binding domain to a DNA nuclease domain; used in gene-editing or genome-editing technologies

TA modules see **toxin-antitoxin modules**

TAT see **twin-arginine transport system**

TatABC complex (twin-arginine translocase) see **twin-arginine transport (TAT) system**

Taxon (plural taxa) one or more populations of organism or organisms that are grouped or classified into a unit that shares certain characteristics, features, genetic similarity, or other relatedness; a taxonomic group of any rank, such as phylum, genus, species; see also **operational taxonomical unit (OTU)**

T-boxes see **t-box riboswitch**

T-box riboswitch a tRNA-mediated mechanism that controls amino acid biosynthesis and aminoacyl tRNA synthetase expression in gram-positive bacteria

TcdA one of the large AB-type protein toxins produced by *Clostridium difficile* that catalyzes the monoglucosylation of small Rho GTPases (also referred to as *C. difficile* toxin A); responsible for symptoms of pseudomembranous colitis

TcdB one of the large AB-type protein toxins produced by *Clostridium difficile* that catalyzes the monoglucosylation of small Rho GTPases (also referred to as *C. difficile* toxin B); responsible for symptoms of pseudomembranous colitis

TcdC a regulatory protein that acts as an anti-sigma factor that inhibits synthesis of *Clostridium difficile* toxins A and B

TcdR an alternative sigma factor that mediates transcription of toxin genes of *Clostridium difficile*

T cell-independent antibody response the activation of B cells by nonpeptide antigens, such as polysaccharides, lipids, or nucleic acids, without interaction with T cells that leads to production of antibodies (without memory); absent in infants

T cell-independent antigen an antigen, such as a polysaccharide, lipid, or nucleic acid, that interacts directly with B cells, bypassing APCs and T cells and directly stimulating an antibody response; no memory cells are produced

T cell epitopes an antigenic determinant that specifies a part of the antigen recognized by antibodies and T cells; groups of amino acids that are located near each other on a protein's surface that can determine antigenicity through binding to the immunoglobulin or antibody

T cell receptor (TCR) a protein complex on the surface of T cells that recognizes a specific epitope bound to an MHC complex

TcpH one component of a two-component regulatory system that along with TcpP is involved in regulating the expression of the *toxT* and *tcp* genes of *Vibrio cholerae*

TcpP one component of a two-component regulatory system that along with TcpH is involved in regulating the expression of the *toxT* and *tcp* genes of *Vibrio cholerae*

TCR see **T cell receptor**

TCS see **two-component regulatory system**

TCT see ***Bordetella* tracheal cytotoxin**

Teichoic acids polysaccharides of glycerol phosphate or ribitol phosphate molecules linked via phosphodiester bonds that are found interwoven with and covalently attached to the peptidoglycan of gram-positive bacteria; they represent as much as 50% of cell wall material

Teicoplanin a glycopeptide antibiotic that inhibits cell wall biosynthesis

Teixobactin a bacterial peptide-like secondary metabolite that kills some Gram-positive bacteria by binding to lipid II and lipid III and inhibiting bacterial cell wall biosythesis

Telithromycin a member of the ketolide family of antibiotics; the only member on the market

Temperate bacteriophages (also temperate phage) see **lysogenic bacteriophage**

Temperate (lysogenic) phages see **lysogenic bacteriophage**

TeNT see **tetanus neurotoxin**

Terminal restriction fragment length polymorphism (T-RFLP) a PCR-based method for profiling microbial communities in which the 16S rRNA gene is PCR amplified using primers to each end that are labeled with a different fluorescent dye; the resulting PCR product is cleaved with restriction enzymes, and the fragments from each end are separated by size using a DNA sequencer

Tetanus spastic paralysis caused by tetanus toxin

Tetanus neurotoxin (TeNT) an AB-type neurotoxin produced by *Clostridium tetani* that has zinc-dependent metalloprotease activity and causes spastic paralysis by cleaving synaptobrevins and preventing neurotransmitter release

Tetracycline destructases enzymes that inactivate tetracycline antibiotics through covalent modification and mediate tetracycline resistance

Tetracyclines a family of antibiotics that bind to 16S rRNA in the 30S ribosomal subunit and distort the A site

Tetrahydrofolic acid an essential cofactor in the biosynthetic pathway leading to formyl-methionine and nucleic acid precursors; synthesis is inhibited by trimethoprim and sulfonamide antibiotics

tetX a gene encoding an enzyme that modifies tetracycline under aerobic conditions

TGGE see **denaturing/thermal gradient gel electrophoresis**

Th cells see **T helper (Th) cells**

Th0 cells see **T helper (Th) cells**

Th1 cells see **T helper (Th) cells**

Th17 cells see **T helper (Th) cells**

Th2 cells see **T helper (Th) cells**

Thermostable riboswitches riboswitches that regulate translation by masking ribosomal binding sites at low temperature and thereby preventing protein translation

Thiry-Vella loop a surgically isolated loop of the intestine that is attached to the abdominal surface; used for studying intestinal secretions and toxin-mediated edema or diarrheal responses

Thymus-dependent antigens antigens that require T cells, which mature in the thymus to activate B cells

Tigecycline a tetracycline derivative with a bulky glycyl-glycine side chain

Tight adherens junctions (or zonula occludens) closely associated areas where epithelial cell membranes join together to form a tight barrier that prevents fluids from moving between the lumen and substratum

Tissue factor (TF, also platelet tissue factor) a protein that initiates blood clotting by cleavage of the zymogen prothrombin into active thrombin

Tissue plasminogen activator (tPA) a protein that activates plasminogen to produce plasmin, which results in an increase in fibrinolytic activity and dissolving of blood clots

TLR1 a toll-like receptor that recognizes peptidoglycan and triacyl lipoproteins from Gram-positive bacteria; works in concert with TLR2 as a heterodimer

TLR2 a toll-like receptor that recognizes lipoteichoic acid, atypical LPS and lipoproteins from Gram-positive bacteria; works in concert with TLR1 as a heterodimer

TLR3 a toll-like receptor that recognizes poly(IC) double-stranded RNA

TLR4 a transmembrane signaling receptor that recognizes LPS bound to LBP and MD-2 to trigger innate immune responses

TLR5 a toll-like receptor that recognizes flagellin

TLR6 a toll-like receptor that works in conjunction with TLR2 to recognize bacterial lipoproteins

TLR7 a toll-like receptor that recognizes single-stranded RNA

TLR8 a toll-like receptor that recognizes GU-rich and G-rich single-stranded RNA

TLR9 a toll-like receptor that recognizes CpG oligonucleotides

TLR10 a toll-like receptor that acts as a suppressor of inflammatory signaling

TLRs see **Toll-like receptors**

Tn-Seq technology (also Illumina Tn-Seq technology, transposon sequencing, or **transposon insertion sequencing)** approach that combines transposon insertional mutagenesis with massively parallel (high-throughput) DNA sequencing technologies of the transposon insertion sites to generate a library of bacterial mutants that can subsequently be used in an infection model or other test system to identify genes contributing to function of interest in the bacteria

TNF-α see **tumor necrosis factor alpha**

Tolerance (dormancy) the dormant (nongrowing) condition where bacteria are able to avoid being killed by antibiotics; bacteria begin growing again when the antibiotic is removed

Toll a transmembrane receptor in *Drosophila* required for resistance to fungal infections

Toll-like receptor 4 see **TLR4**

Toll-like receptors (TLRs) a group of mammalian surface proteins that interact with various PAMPs (LPS, peptidoglycan fragments, and other microbial components) and signal innate immune responses, such as changes in cytokine production

Toxic shock see **toxic shock syndrome**

Toxic shock syndrome (TSS) a potentially lethal disease caused by strains of *Staphylococcus aureus* that produce the superantigen toxic shock syndrome toxin 1 (TSST-1); symptoms include fever, rash, exfoliation of the palms and soles of the feet, and toxic shock

Toxic shock syndrome toxin (TSST) see **Toxic shock syndrome toxin 1**

Toxic shock syndrome toxin 1 (TSST-1) an exotoxin produced by some strains of *Staphylococcus aureus*; a superantigen

Toxin A see **TcdA**

Toxin B see **TcdB**

Toxin neutralization the binding of antibodies to toxins, thereby preventing toxin action, such as binding of the toxin to host target cells

Toxin-antitoxin (TA) systems two-component protein complexes consisting of a toxin that, when expressed by itself, shuts down various cellular processes, including those susceptible to antibiotics, and an antitoxin that binds tightly to the toxin and prevents toxin action; may allow persisters to survive in environments containing antibiotics

Toxoid a protein toxin that has been chemically or heat treated to destroy its toxicity but retain its immunogenicity; the inactive fragment or recombinant form of a protein toxin

Toxoid vaccine a vaccine that consists of toxoids (inactivated toxins); examples are diphtheria vaccine and tetanus vaccine

ToxR a component of the regulatory system that, along with ToxS, controls the expression of at least 17 genes in *Vibrio cholerae;* regulated genes include those for toxin, pili, serum resistance, and outer membrane proteins

ToxS a component of the regulatory system that, along with ToxR, controls the expression of at least 17 genes in *V. cholerae*; regulated genes include those for toxin, pili, serum resistance and outer membrane proteins

ToxT a global regulator that activates the expression of *tcp, ctxAB,* and other virulence genes in *Vibrio cholerae;*

its synthesis is regulated by ToxR, ToxS, TcpP, and TcpH

ToxT regulon a regulon containing *tcp, ctxAB,* and other genes located in different places on the *Vibrio cholerae* chromosome that are regulated similarly by ToxT

tPA see **tissue plasminogen activator**

tracrRNA a small trans-encoded trans-activating crRNA involved in CRISPR-Cas systems that base pairs with the crRNA to form a functional guide RNA, which binds to Cas9 and to the CRISPR spacer sequence

Transconjugants the progeny that result from conjugation

Transcription factor a regulatory protein that binds to specific bacterial gene promoters and changes transcript levels

Transcriptional activator a regulatory protein that facilitates binding of RNA polymerase to promoters and increases initiation of transcription; a positive regulator

Transcriptional antiterminators factors that allow RNA polymerase to resume transcription following premature transcription termination

Transcriptional fusion a hybrid gene with the promoter/operator of one gene (such as a virulence gene) fused to a promoterless reporter gene encoding an assayable enzyme or fluorescent protein

Transcriptional repressor a protein that prevents transcription of genes by binding to an operator

Transcriptional response the global changes in transcription, in terms of relative amounts determined by microarrays, in bacterial or host cells in response to adherence, invasion, or other stimuli

Transcriptional terminators Rho factor-independent RNA secondary structures (hairpin loops) followed by a run of uridine residues that cause RNA polymerase to terminate transcription

Transcriptome the full complement of RNA transcripts determined by relative transcript amounts for each gene in an organism

Transcriptomics the study of RNA transcripts that are produce under specific conditions in a cell or in a population of cells

Transcytosis a type of transcellular transport where macromolecules are captured in vesicles and transported from one side of the cell to another side

Transduction the transfer of genetic information from one bacterial cell to another by a bacteriophage

Transfection a process by which nucleic acids are introduced into mammalian cells

Transferrin a blood and tissue glycoprotein synthesized by the liver that binds tightly to and sequesters iron

Transformant the progeny resulting from DNA transformation

Transformation a process in which competent bacteria take up free DNA from the environment

Transgenic animals animal strains with specific genetic alterations that are used in infection models of disease

Transgenic mice mouse strains with specific genetic alterations; examples are knockout mice and knock-in mice

Transglycosylation a process by which *N*-acetylmuramic acid and *N*-acetylglucosamine residues of peptidoglycan are linked to form the polysaccharide glycan backbone

Translational attenuation a type of regulation that controls the expression of genes involved in imparting resistance to translation-inhibiting antibiotics, such as erythromycin

Translational fusion a gene fusion where two genes are joined in frame to generate a gene encoding a fusion protein; can be used to measure virulence gene expression levels or promoter activity where a reporter gene is fused to the promoter of a virulence gene

Translocase complex a part of the general secretory pathway; part of a protein-conducting channel through the cell membrane

Translocation the movement of the catalytic A subunit of an AB-type toxin into the cytoplasm of a host cell

Translocon a complex of proteins associated with translocation of polypeptides across membranes

Transmigration the movement of natural killer cells and PMNs across the blood vessel wall into tissues

Transmission the spread of infectious disease through direct contact with an infected individual or directly through contact with a vector or other fomite that is contaminated with the infectious agent

Transpeptidation the formation of peptide bonds between peptidoglycan peptides that link different peptidoglycan chains

Transposase an enzyme that catalyzes the transposition of transposons and insertion sequences

Transposon a segment of DNA containing insertion sequences flanking one or more genes encoding a transposase and virulence genes or antibiotic resistance genes

Transposon insertion mutagenesis an approach by which a transposon is randomly inserted into a bacterial chromosome, thereby interrupting or modifying the function of the disrupted gene and causing a mutation; used to screen for the biological function of the disrupted gene (e.g., its role in infection)

Transposon mutagenesis see **transposon insertion mutagenesis**

Treg cells see **T helper (Th) cells**

Triclosan an antiseptic/disinfectant incorporated into many plastic products

Trimethoprim a bacteriostatic antibiotic that inhibits an enzyme in the tetrahydrofolic acid biosynthetic pathway

Tropism a biological phenomenon indicating the growth, orientation, or movement of a microbe toward an external stimulus

TSS see **toxic shock syndrome**

TSST see **toxic shock syndrome toxin 1**

Tumor necrosis factor alpha (TNF-α) a proinflammatory cytokine produced by monocytes and macrophages in response to LPS

Twin-arginine translocase see **twin-arginine transport (TAT) system**

Twin-arginine transport (TAT) system (also Twin-arginine translocase) a transmembrane protein export system dedicated to the export of fully folded proteins out of the cell; found in both gram-positive and gram-negative bacteria; a protein transport complex comprised of the proteins TatA, TatB, and TatC

Two-component regulatory system (TCS) a bacterial signal transduction system in which one protein senses the signal and then phosphorylates the second protein to produce the form that responds to the signal, often regulation of transcription

Two-dimensional (2D) gel electrophoresis a proteomics technique used to separate proteins within complex mixtures based on their biochemical and electrophoretic properties; in the first dimension, proteins are separated based on their pI value using an isoelectric focusing gel, and in the second dimension, they are separated based on their relative size using an SDS-PAGE gel

Type I toxin a bacterial toxin that is not translocated into a host cell but binds to the target cell surface; an example is superantigens

Type II toxin a bacterial toxin that acts on the host cell membrane; examples are phospholipase and pore-forming cytotoxins

Type III toxin a classical AB-type toxin that has a binding region that recognizes a specific host cell receptor, a translocation region that mediates translocation across the cell membrane, and a catalytic toxic domain that enters the host cell cytoplasm and modifies a host protein, rRNA, signaling molecule, metabolite, or other host cell process

Type 7 secretion system (T7SS) specialized secretion system present in Gram-positive bacteria, such as *Staphylococcus aureus, Mycobacteria,* and *Streptomyces coelicolor,* comprised of a membrane-bound hexameric ATPase and any one of the EsxA/EsxB-related proteins or the Ess system; secretes Esa effector proteins

uidA a gene encoding *Escherichia coli* β-glucuronidase (GUS); a reporter gene

Uni ProtKB Universal Protein Resource Knowledgebase; a database that provides protein translations of nucleotide sequences from the nucleotide sequence databases

Unidentified reading frame (URF) a gene sequence encoding a putative polypeptide of unknown function that has no homology to known proteins; see **open reading frame**

United States Department of Agriculture (USDA) a federal agency responsible for developing and executing federal laws related to farming, forestry, and food; for providing guidance about food, agriculture, natural resources, rural development, and nutrition

UPEC see **uropathogenic *Escherichia coli* strains**

URF see **unidentified reading frame**

Urinary tract infection (UTI) an infection of any portion of the urinary tract

Uroepithelium the epithelial cells lining the urinary tract

Uropathogenic *Escherichia coli* (UPEC) strains strains of *E. coli* that cause urinary tract infections

Uroplakins mannose-containing glycoproteins located on the luminal surfaces of bladder epithelial cells that are the sites of attachment of UPEC strains

Usher a protein dimer in the outer membrane that aids in the assembly and export of pilin

UTI see **urinary tract infection**

Vaccination the stimulation of an adaptive immune response to a specific pathogen or antigen by administering a vaccine

Vaccine a suspension of microorganisms (usually killed or attenuated) and/or their products (toxoids, adhesins, and surface proteins), usually including an adjuvant, that is used for immunization

Vacuolating toxin (VacA) a secreted T5SS autotransporter protein toxin produced by Helicobacter pylori that binds and enters host cells and forms anion-selective channels in membranes, resulting in severe vacuolation characterized by accumulation of large vesicles

Vaginal-associated lymphoid tissue (VALT) mucosal associated lymphoid tissue found in the vagina that supports local immune priming of T cell and B cell immune responses

Vaginitis inflammation of the vagina that results in discharge, itching and pain; usually resulting from fungal (yeast) infection

Vaginosis disease of the vagina cause by microbial dysbiosis (particularly loss of lactobacilli) that results in a fishy-smelling discharge, and can increase risk for other sexually transmitted diseases, pelvic inflammatory disease, and preterm birth and other pregnancy complications

Valence the number of antigen- or ligand-binding sites available for binding epitopes on an antigen or ligands on a receptor

VAMP see **synaptobrevin**

***vanA* and *vanB* genes** vancomycin resistance genes that encode an enzyme that makes ᴅ-Ala-ᴅ-lactate

Vancomycin a glycopeptide antibiotic that inhibits bacterial cell wall biosynthesis

Vancomycin-resistant *Enterococcus* (VRE) strains of *Enterococcus* (usually *Enterococcus faecium*) that are resistant to vancomycin; a common cause of nosocomial infections

Vancomycin-resistant *Staphylococcus aureus* (VRSA) strains of *Staphylococcus aureus* that have become resistant to the glycopeptide antibiotic vancomycin

***vanH* gene** a vancomycin resistance gene that encodes lactate dehydrogenase, which catalyzes the conversion of pyruvate to lactate

***vanX* gene** a vancomycin resistance gene that encodes an enzyme that cleaves D-ala from D-ala-D-ala

Variable region (Fv) the N-terminal portion of the Fab domain of an antibody molecule that binds to a specific antigen

Variolation smallpox vaccine inoculation method that involve scraping a small amount of material taken from an infected patient into the skin of another in order to cause only a mild, hopefully protective infection

Vascular related to blood vessels of the circulatory system

Vasoactive see **vasoactive compounds**

Vasoactive compounds compounds released by Mast cells that dilate blood vessels and cause vasodilation and decreased blood pressure; mediators of inflammation

Vasodilation the dilation (widening) of blood vessels in response to hormone-induced relaxation of smooth muscle cells within the vessel walls

V(D)J recombination somatic recombination that occurs in developing lymphocytes during the early stages of T cell and B cell maturation

Vector (i) a transmitter of infectious microorganisms, such as an arthropod; (ii) a virus or plasmid used in cloning

Verotoxin an AB-type protein toxin related to Shiga-like toxin (SLT) that is produced by some strains of *Escherichia coli*

Virosome a vesicle composed of a lipid membrane with membrane-bound viral proteins (often hemagglutinin or neuraminidase) that serves as an efficient antigen carrier and adjuvant in vaccine designs

Virotypes the different strains of a particular bacterial species that are grouped based on their virulence factors or virulence properties, such as the different strains of *Escherichia coli*

Virotyping the classification of various strains of a particular species of bacteria based on the types of virulence factors or different disease strategies exhibited

Virstatin a small molecule that inhibits ToxT; discovered by high-throughput phenotypic screening

Virulence the ability of a microorganism to cause disease

Virulence factor a bacterial product or strategy that contributes to the ability of the bacterium to survive in the host and/or cause infection

VISSA see **vancomycin-intermediate susceptibility *Staphylococcus aureus***

VRE see **vancomycin-resistant *Enterococcus***

Warhead the reactive portion of a drug or antibiotic that often forms a covalent bond with its target to inactivate it (e.g., the β-lactam ring found in β-lactam antibiotics)

Waterborne disease see **waterborne infections**

Waterborne infections bacterial infections acquired from contaminated water; diseases caused by drinking contaminated (or dirty) water; caused by a variety of microbes, biotoxins, and toxic contaminants in water

Weapon of mass destruction (WMD) a bioweapon used as an implement in a state-sponsored war conducted on a grand scale

Weapon of mass disruption (WMD) a bioweapon used on a small scale by groups or individuals to spread terror and disrupt normal routine operations

White blood cell see **leukocyte**

Whole-body biophotonic imaging a noninvasive technique for monitoring disease progression in live animals whereby the bacteria (or sometimes animal hosts) are engineered with reporter genes that express bioluminescence when activated during colonization or invasive disease

Whole-genome sequencing (WGS) technologies see **DNA sequencing technologies**

Whooping cough see **pertussis**

WMD see **weapon of mass destruction** or **weapon of mass disruption**

World Health Organization (WHO) a specialized agency of the United Nations that is responsible for dealing with international public health and responses to global health issues

Wound botulism a paralytic disease caused by botulinum neurotoxin produced by *Clostridium botulinum* growing in a wound

XDR see **extensively drug resistant**

X-Gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a chromogenic substrate for β-galactosidase that produces a blue color when cleaved

X rays radiation generated when an electron beam is directed at a thin plate of gold or other metal; used to irradiate food

Yersiniabactin a siderophore produced by pathogenic *Yersinia* species responsible for ferric iron and zinc metal ion acquisition

YidC a membrane protein chaperone that interacts with the Sec translocon of the general secretion system and also acts as a Sec-independent membrane protein insertase

Yops the outer membrane or secreted proteins of *Yersinia;* some are part of a type 3 secretion system (T3SS), and others are T3SS effector proteins that are delivered directly to eukaryotic cells and cause toxic effects on the host cells

ZFN (zinc finger nuclease) engineered restriction enzymes generated by fusing a zinc-finger DNA-binding domain with a DNase domain; used in gene-editing or genome-editing technologies

Zinc-dependent β-lactamase a type of β-lactamase that can cleave many classes of β-lactams and is not inhibited by clavulanic acid; does not contain a serine residue in its active site

Zonula occludens see **tight adherens junctions**

Zoonotic disease (also Zoonosis) an infectious disease primarily spread in animals that can also be transmitted to humans

Zymogen a proenzyme that is inactive, but can be converted into an active enzyme by modification via another enzyme (usually a protease)

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